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A Simple RP-HPLC Bio Analytical Method for Determination of Levetiracetam in Human Serum

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ABSTRACT

A simple, precise, accurate and linear reverse-phase high-performance liquid chromatography method using UV detection for the estimation of the novel antiepileptic, Levetiracetam was established and validated. A Simple protein precipitation method along with acetonitrile as precipitating solvent was used for the extraction of Levetiracetam from healthy human volunteers. HPLC analysis was carried out on a C18 (4.6mm*250mm, 5µm), column. The mobile phase consisted of a composition of ammonium acetate buffer (10mM, pH 5) and acetonitrile (50:50v/v) with an isocratic flow rate of 0.3mL/min over 15min runtime. Chromatograph was read at 205 nm. The retention time through this method was recorded as 7.8 min for Levetiracetam and 9.2 min for Fluconazole (internal standard). The detector response was ruled out to be linear in the concentration of 10-50 µg/mL with a mean correlation coefficient of 0.99. The limit of detection and limit of quantification were noted as 0.8µg/mL and 2.5µg/mL, respectively. The percent RSD for precision was within the acceptance criteria of not more than 2.0%. The Bio analytical Method developed above was found to be precise, accurate and linear within its therapeutic dose. This makes the method widely applicable for the regular analysis of Levetiracetam in the bio analytical matrix for toxicity or therapeutic drug monitoring.

KEY WORDS: Levetiracetam, RP-HPLC, UV detection, protein precipitation, RSD.

1. INTRODUCTION

Levetiracetam [S-enantiomer of α-ethyl-2-oxo-1-pyrrolidine acetamide; Keppra] (Fig.1) is an antiepileptic drug (AED) which is structurally and mechanistically different from other antiepileptic drugs (Hovinga, 2001). It is FDA approved drug used to treat patients with partial onset seizures, myoclonic seizures (Schachter, 2000; Nash and Sangha, 2001; Dooley and Plosker, 2000) primary generalized tonic-clonic seizures. Levetiracetam has a favorable pharmacokinetic profile; after oral intake Levetiracetam absorption was rapid and complete (Tmax< 1hour, its bioavailability is close to 100%), plasma protein binding is low (<10%), insignificant hepatic metabolism, is not metabolized by CYP-dependent pathways that produces limited drug-drug interactions, rapid attainment of steady-state concentrations, excretion is primarily renal; approximately 66% of the dose found unaffected in urine and 24% is excreted in urine as its acid metabolite form. The metabolite which excreted in urine was pharmacologically inactive. The half-life of elimination of oral Levetiracetam is between 6 to 8 hours in grown-ups (Iwasaki, 2015; Patsalos, 2004) and 5-7hrs in children (Wright, 2013). The primary adverse effects are CNS related and include a headache, asthenia, somnolence, and dizziness. Levetiracetam has better efficacy comparable to other new anti-epileptic drugs (McAuley, 2002) and it has a wide therapeutic window, where toxic doses are well differentiated from therapeutic dosages (Patsalos, 2000). The efficacy is concentration dependent (Perucca and Bialer, 1996; Pellock, 2001; Boon, 2002). For the monitoring of drug concentration serum was used as a medium.

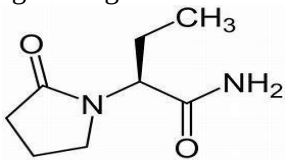

Figure.1.Chemical structure of Levetiracetam

2. MATERIALS AND METHODS

Chemicals and Apparatus: Levetiracetam was procured from Hetero Drugs Limited. Fluconazole (used as internal standard), ammonium acetate, HPLC grade water, and acetonitrile were procured from Sigma Aldrich, Mumbai. High-Performance Liquid Chromatographic system (Shimadzu's LC 20AD) typically consists of a 25µL fixed volume injector (Rheodyne). The chromatographic separation of Levetiracetam & Fluconazole (internal standard) was performed on C18 (4.6mm*250mm, 5µm), column using UV-visible detector SPD-20A.

Ethical Approval: IHEC approval was obtained after submission of protocol IHEC ECR/257/Indt/TG/2015/VCOP / MGMH /PHARM D/2017/003.

Calibration Samples: Stock solutions of Levetiracetam and Fluconazole were set up by dissolving the appropriate amount in acetonitrile to yield 1mg/mL drug concentration. The dissolution was accelerated by sonication of the mixture for 10 min. The dissolved solutions were stored at -20ºC. Calibration samples in the concentration range from 10-50µg/mL were obtained by spiking human serum samples which are drug-free along with the working solution. This range covers the therapeutic concentrations of Levetiracetam in patient samples.

Human Serum Sampling: Frozen, drug-free plasma (blank plasma) for calibration curves was obtained from healthy subjects stored at -20ºC and thawed at room temperature before use. Venous blood samples were drawn from healthy volunteers, transferred into serum separator tubes and centrifuged at 4ºC at 3000rpm using REMI centrifuge for 10 min. Serum was separated and stored at -4ºC.

Sample Extraction: To 250µL serum sample previously spiked with an analyte (of varying concentrations 1050µg/mL) and fluconazole (internal standard), an equivalent amount of acetonitrile was added. The mixture was vortexed for 1min on a cyclomixer, which was further centrifuged at 3000rpm for 10min at 4ºC. The supernatant was collected carefully and the sample is sterile filtered using a syringe filter of 0.2µm pore size and the sample is injected into the HPLC instrument.

Chromatographic Conditions: After the injection of 20µL of the prepared sample, the separation of Levetiracetam was accomplished by isocratic elution. The mobile phase consists of ammonium acetate buffer (10mM) - acetonitrile (50:50v/v), and (pH*5 ± 0.2). The mobile phase was filtered with a 0.45µm filter before loading on to the column. The flow rate of the mobile phase was set to 0.3mL/min with a run time of 15 min. Chromatograph was read at 205 nm wavelength using detector UV-visible SPD-20A. The retention time of Levetiracetam and Fluconazole (internal standard) was 7.8 min and 9.2 min respectively. The chromatogram of standard solution shown in Figure.2.

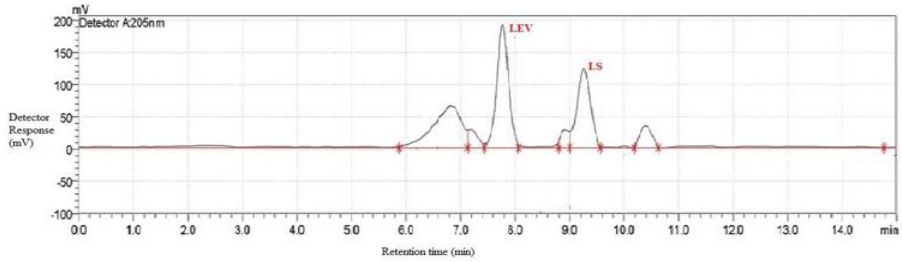

Figure.2. Chromatogram of standard solution containing Levetiracetam and I.S (Fluconazole)

Method Development: Method development was executed according to ICH conditions for specificity, the range of linearity, accuracy, precision and robustness.

3. RESULTS AND DISCUSSION

System Suitability: System suitability test is performed in order to know if the resolution and reproducibility of the chromatographic systems are satisfactory. The evaluation tests primarily depend on the equipment, electronics, and samples to be examined in an integral system. The limits for system suitability were being set for Theoretical plates, Resolution, Asymmetry. Five injections of the standard mix and two injections of the sample were injected for this purpose. The Resolution, Areas, Retention time, Theoretical plate's values and peak Asymmetry were calculated for standard and sample solutions. System suitability results were shown in Table.1. The results obtained were considered to be in acceptable limits (Tailing factor ≤2.0 and Theoretical plate's ≥2000).

Table.1. System suitability parameters

Mobile phase	10mM ammonium acetate: acetonitrile 50:50v/v	Wavelength	205 nm
Pump mode	Isocratic	Injection volume	20 µL
pH	5	Flow rate	0.3 mL/min
Diluents	Acetonitrile	Run time	15 min
Column	Chromosil C18 column (250 X 4.6 mm, 5µm)	Retention time	7.8 min
Column temperature	Ambient		

Linearity: The linearity was found at ten concentration levels ranges between 10µg/mL-50µg/mL. The graph of the peak area of the individual sample with respect to the concentration of Levetiracetam was found to be linear in the range of 10-50 µg/mL. The correlation coefficient (r) of the standard curve was established as 0.9987. Linearity results were shown in Table.2.

Table.2. Linearity results

Concentration of Levetiracetam	Peak height	Concentration of Levetiracetam	Peak height
10	1,82,965	50	6,13,067
20	2,65,683	Slope	10915
30	3,65,347	Intercept	63854
40	4,96,931	Correlation coefficient	0.99

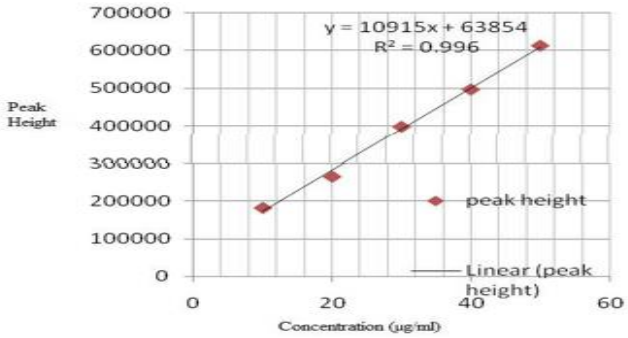

Figure.3. On X axis concentration of sample, On Y axis peak height response

Precision: The precision is an analytical parameter which generates reproducible results by the predefined method. This parameter could be determined by repeatability (intraday) and intermediate precision (inter-day) the value derived is considered as %RSD. By using this method six Levetiracetam (ppm) replicate standard solutions were prepared and thereafter analyzed using the predefined method. It was reported that percent relative standard deviation as 0.02% for peak responses which would be good within the accepted criteria of not more than 2.0%. Results of precision study were shown in Table.3.

Table.3. Precision results for Levetiracetam

Sample	Concentration of Levetiracetam	Injection No.	Peak areas	RSD (Acceptance criteria <2.0%)
Levetiracetam	10	1	182965	0.02
		2	182972	
		3	182899	
		4	182929	
		5	182963	
		6	182969	

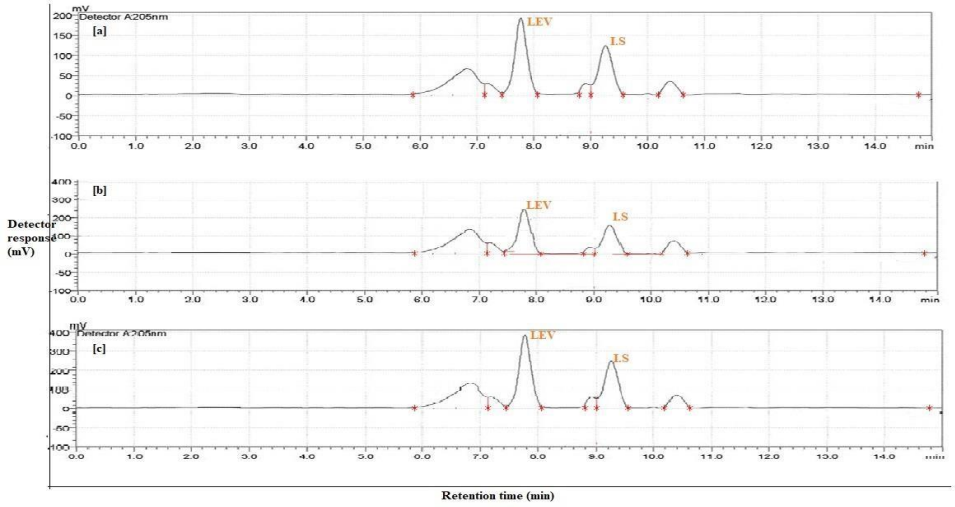

Figure.4. Chromatograms obtained by injecting blank plasma spiked with (a) LEV, 20µg/mL and I.S., 10µg/mL; (b) LEV, 30µg/mL and I.S., 10µg/mL; and (c) LEV, 40µg/mL and I.S., 10µg/mL LEV, Levetiracetam; Fluconazole [I.S-internal standard]

Limit of Detection (LOD) and Limit of Quantitation (LOQ): Limit of Detection (LOD) is defined as the lowest concentration of analyte which gives a detectable response. Limit of quantitation is defined as the lowest concentration that can be quantified reliably with a specified level of accuracy and precision. By using mobile phase sample was dissolved and continued injecting till peak got disappeared. The LOQ and LOD results were given in Table.4.

Table.4. Limit of Detection and Limit of Quantification

Parameters	Results
LOD	0.8µg/mL
LOQ	2.5µg/mL

Robustness: Robustness of the assay was evaluated by considering typical variations in conditions of liquid chromatography. The robustness check was performed by altering the flow rate, the pH of the solution, and the method was found to be robust. Results of robustness study were shown in Table.5.

Table.5. Robustness results for Levetiracetam

S.No.	Parameter	Condition	Area
1	Standard	Standard Conditions	1,82,965
2	Flow rate	0.2mL	1,83,000
3	pH of mobile	4.8	1,82,992
	phase at	5.2	1,82,589

Accuracy: By analyzing the Quality control samples extraction recovery of the analyte and internal standard from plasma was evaluated. Recovery at three concentrations was determined by comparing peak areas obtained from the plasma samples with those from the standard solution spiked with the blank plasma residue (75%, 100%, and 125%). The mean recovery values of 75%, 100% and 125% spiked samples were reported as 102%, 99%, and 103% respectively.

4. CONCLUSION

The Bio analytical Method developed was found to be precise, accurate and linear within its therapeutic dose. This makes the method widely applicable for the regular analysis of Levetiracetam in the bio analytical matrix for toxicity or therapeutic drug monitoring (TDM).

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