Research Article

AN IMPROVED RP-HPLC METHOD FOR THE QUANTITATIVE DETERMINATION AND VALIDATION OF LEVETIRACETAM IN BULK AND PHARMACEUTICAL FORMULATION

Ravisankar P*, Niharika A, Srinivasa Babu P

Department of Pharmaceutical Analysis and Quality Assurance, Vignan Pharmacy College, Vadlamudi, Guntur, Andhra Pradesh, India

*Corresponding Author Email: banuman35@gmail.com

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ABSTRACT

The present study depicts the development of a validated RP-HPLC method for the determination of the Levetiracetam in pharmaceutical tablet dosage form. RP-HPLC method was developed by utilizing Welchrom C₁₈ Column (250 x 4.6 mm, 5μ), Shimadzu LC-20AT Prominence Liquid Chromatograph. The mobile phase composed of 10 mM Phosphate buffer: acetonitrile (50:50 v/v) (pH-3.0, adjusted with triethylamine). The flow rate was set to 1.0 ml/min with the responses measured at 243.2 nm using Shimadzu SPD-20A Prominence UV-Visible detector. The retention time of Levetiracetam was found to be 3.030 minutes. Linearity was established for Levetiracetam in the range of 10-50 μg/ml with correlation coefficient 0.9998. The LOD and the LOQ for Levetiracetam found to be 0.468 μg/ml and 1.421 μg/ml respectively. The amount of Levetiracetam present in the formulation was found to be 99.56 %. None of the excipients interfered with the analyte of interest. Considering all the results of validation parameters simplicity of the method and the cost effectiveness of the overall procedure, it is possible to conclude that the developed method can be suitable for the regular quality control determination of Levetiracetam in bulk as well as pharmaceutical dosage form.

Keywords: Levetiracetam, RP-HPLC, Determination, Validation, Pharmaceutical dosage form.

INTRODUCTION

Levetiracetam^{1,2} is a novel antiepileptic drug³ recently approved by the U.S. Food and Drug Administration as or as an adjunct in partial, myoclonic and tonic-clonic seizures and mono therapy for partial seizures with or without secondary generalization. Levetiracetam has potential benefits for other psychiatric and neurologic conditions⁴ such as Tourette syndrome, autism, and anxiety disorders. Levetiracetam seems to be a safe and effective treatment for migraine⁵ with aura. Chemically it is (αS)-α-ethyl-2-oxo-1-pyrrolidineacetamide with a molecular formula of C₈H₁₄N₂O₂ and a molecular weight of 170.20 g/mol. This is a structural analog of piracetam, which binds to a synaptic vesicle protein SV2A and is believed to impede nerve conduction across synapses. The exact mechanism by which Levetiracetam shows its antiepileptic effect is still unknown. However, it is believed that it binds to a synaptic vesicle protein, thus slowing down nerve conduction across synapses. Stability studies suggest proper formulations, design manufacturing processes, and selecting proper storage condition and packaging for the drug product. Furthermore, it helps in establishing shelf life of product⁶⁻⁹. All these make it highly important to have a stability indicating method in hand. Literature review reveals that various analytical methods in pure and pharmaceutical dosage forms like UV spectrophotometry¹⁰, HPLC-UV¹¹⁻¹⁸, Capillary electrophoresis¹⁹ and LC-MS²⁰⁻²² and other analytical methods have been developed individually. HPLC-UV is the most commonly available sophisticated technique, utilized to develop quantification of Levetiracetam. The objective of the current study was to develop an accurate, simple, and economical analytical method for the estimation of Levetiracetam in pharmaceutical dosage forms. However the requirement of very simple, fast, efficient, precise, time saving and highly reliable analytical RP-HPLC method for routine quality control purpose always necessities to see a new and better method. Hence, it was proposed to develop a simple, trouble-free, fast, perfect,

and sensitive HPLC method with commonly available UV detector for the concurrent estimation of Levetiracetam in pure form and pharmaceutical formulations. The structural formula of Levetiracetam is shown in Figure 1.

MATERIALS AND METHODS

Chemicals and Reagents

Levetiracetam standard reference drug was kindly supplied as gift sample by Hetero Drugs Ltd., Hyderabad, and Andhra Pradesh, India. All the chemicals were analytical grade from Rankem Ltd., Mumbai, India, while acetonitrile (HPLC grade) Potassium dihydrogen phosphate dihydrogen Potassium hydrogen phosphate and triethylamine (HPLC grade) procured from Merck Pharmaceuticals Private Ltd., Mumbai, India. Ortho phosphoric acid used was of HPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India. Commercial tablets of Levetiracetam formulation was procured from local market. Kepra tablets 500 mg are manufactured by USB India Pvt. Ltd., Mumbai.

Instrumentation

Quantitative HPLC was performed on a isocratic high performance liquid chromatography (Shimadzu LC-20AT Prominence Liquid Chromatograph) with a LC-20AT VP pump, manual injector with loop volume of 20 μL (Rheodyne), programmable variable wavelength Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C₁₈ Column (4.6 X 250 mm, 5μm particle size). The HPLC system was equipped with "Spinchrome" software. In addition an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model-2203) were used in this study.

Chromatographic conditions

Chromatographic separation was performed on a Shimadzu LC-20AT Prominence Liquid Chromatograph, injection system (LC-20AT VP pump, manual injector with loop volume of 20 μ L (Rheodyne system 20 μ L loop). An ODS C₁₈ RP-column (250 mm X 4.6 mm, 5 μ m) was selected. The elution was carried out isocratically at flow rate of 1 ml/min using the mixture of 10 mM Phosphate buffer (pH adjusted to 3.0 using triethylamine) and acetonitrile in ratio of 50:50 v/v was selected as mobile phase.

Mobile phase preparation

Accurately weighed 1.488 g of KH₂PO₄ and 0.288 g of K₂HPO₄ dissolved in 500 ml of water to get phosphate buffer. pH was adjusted to 3.0 with triethyl amine. Above prepared buffer and acetonitrile were mixed in the proportion of 50:50 v/v. This mixture was sonicated for 10 minutes and filtered through 0.22 μ m membrane filter and used as mobile phase.

Preparation of standard stock and working standard drug solution

Accurately weighed 10 mg of Levetiracetam was transferred to 100 ml volumetric flask. The volume was made to the mark with mobile phase to obtain stock solution of Levetiracetam 100 μ g/mL. From this solution prepared working range concentrations. The above prepared solution was further diluted with the mobile phase in different calibrated flasks to obtain working standard solutions of suitable linearity range concentrations (10 - 50 μ g/ml) of Levetiracetam.

Preparation of Sample solutions

The content of 20 tablets of Keppra and Epictal were exactly weighed separately and transferred into a mortar and ground to a fine powder separately. From these, a tablet powder which is equivalent to 100 mg of Levetiracetam was taken and the drugs were extracted in 100 mL of mobile phase separately. The resulting solution was Levetiracetam filtered using 0.22 μ m membrane filter paper and degassed by sonication. These solutions were further suitably diluted for chromatography.

Selection of detection wavelength

For the selection of analytical wavelength 10 μ g/mL Levetiracetam solution was prepared from standard drug solution and scanned in the range of 200 to 400 nm. From the UV spectra, the maximum λ_{max} of Levetiracetam is found to be 243.2 nm. So this wavelength was selected as the detection wavelength for analysis.

Optimization of mobile phase

Optimization of mobile phase was performed based trial and error method. In this different mobile phase trial was taken like in methanol:water, ACN:water and methanol:ACN:water in different ratio without pH but there are different problem were observed like high tailing factor value and not optimized theoretical plate. When the mixture of 10 mM Phosphate buffer (pH adjusted to 3.0 using triethylamine) and acetonitrile in ratio of 50:50 v/v was selected as mobile phase Levetiracetam full fill all the criteria of system suitability. The mobile phase consisting of 10 mM Phosphate buffer (pH adjusted to 3.0 using triethylamine) and acetonitrile in ratio of 50:50 v/v was selected which gave sharp peak with retention time of 3.030 min. So finally the above said mobile phase was selected for analysis. Optimized chromatographic conditions and system suitability parameters are shown in Table 1.

Calibration curve Levetiracetam

Replicates of each calibration standard solutions (10, 20, 30, 40, 50 μ g/mL) were injected utilizing a 20 μ L fixed loop system and the chromatograms were recorded. Calibration curves were constructed by plotting concentration of Levetiracetam on X-axis and peak areas of standard Levetiracetam on Y-axis. The least square analysis method was followed for attaining the slope, intercept and correlation coefficient, regression data values. The calibration data is presented in Table 2. Linear regression data of the proposed method of Levetiracetam is computed in Table 3. The calibration curve of Levetiracetam is shown in Figure 2.

VALIDATION OF THE PROPOSED METHOD

The developed method of analysis was validated as per the ICH Q2 (R1) guide lines²³ for the different parameters like linearity, specificity, precision, accuracy, robustness and limit of detection (LOD) and limit of quantitation (LOQ). Suitability of chromatographic system was tested before validation. Five replicate injections of standard preparation were injected and Retention time, tailing factor, number of theoretical plates, and relative standard deviation of peak area were determined.

Specificity

The effect of wide range of excipients and other additives usually present in the formulations of Levetiracetam in the determinations under optimum conditions was investigated. The specificity of RP-HPLC method was established by injecting the mobile phase and placebo solution in triplicate and recording the chromatograms. The common excipients such as lactose anhydrous, microcrystalline cellulose and magnesium stearate have been added to the placebo solution and injected and tested. The representative chromatogram of placebo is shown in Figure 3. The specificity results are presented in Table 4

Linearity

The linearity graphs for the proposed assay methods were obtained over the concentration range of 10-50 μ g/mL of Levetiracetam. Calibration data values and the results are presented in Table 2. The representative chromatograms of 10-50 μ g/mL indicating the Levetiracetam are shown in Figure 4 to 8 and the standard chromatogram of Levetiracetam are shown in Figure 9.

Precision

Intra-day and inter-day precision study of Levetiracetam was carried out by estimating corresponding responses 3 times on the same day and on 3 different days for the concentration of 30 μ g/mL. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The results for intra-day and inter-day precision are presented in Table 5 and Table 6 respectively.

Accuracy (Recovery studies)

The accuracy of the method was determined by calculating recovery of Levetiracetam by the method of addition. Known amount of Levetiracetam at 50 %, 100 % and 150 % was added to a pre quantified sample solution. The recovery studies were carried out in the tablet in triplicate each in the presence of placebo. The mean percentage recovery of Levetiracetam at each level was not less than 99 % and not more than 101 %. The results are presented in Table 7.

Robustness

The Robustness was evaluated by the analysis of Levetiracetam under different experimental conditions such as making small changes in flow rate (± 0.2 mL/min), detection wavelength (± 5 nm), and Mobile phase composition ($\pm 5\%$). The results are presented in Table 8.

LOD and LOQ

Limit of detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of detection and Limit of quantitation were calculated using following formula $LOD = 3.3(SD)/S$ and $LOQ = 10$

$(SD)/S$, where SD = the standard deviation of response (peak area) and S = the slope of the calibration curve. The LOD and LOQ values are presented in Table 9.

Application to commercial tablet

Utilizing the developed RP-HPLC chromatographic method, assay of Levetiracetam in tablet was carried out as mention in the experimental section. Six replicate determinations were made. Satisfactory results were obtained and were good agreement with the label claim and assay results were shown in Table 10. The results were very close to the labeled value of commercial tablets. The representative sample chromatogram of Levetiracetam is shown in Figure 10.

Table 1: Optimized chromatographic conditions and system suitability parameters

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C ₁₈ Column (4.6 X 250 mm, 5 μ m)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	10 mM Phosphate Buffer (pH-3): Acetonitrile (50:50 v/v)
Mobile phase	10 mM Phosphate Buffer (pH-3): Acetonitrile (50 : 50 v/v)
Flow rate	1 mL/min.
Detection wave length	By UV at 243.2 nm.
Run time	8 Minutes
Column back pressure	98 kgf
Temperature	Ambient temperature (25°C)
Volume of injection loop	20 μ L
Retention time (R _t)	3.030 minutes
Theoretical plates [th.pl] (Efficiency)	17803
Theoretical plates per meter [t.p/m]	356053
Tailing factor (asymmetric factor)	1.063

Table 2: Calibration data of the proposed HPLC method of Levetiracetam

Concentration, μ g/mL	Retention time, (R _t) min.	Peak area, mV.s.
0	0	0
10	3.023	205.5
20	3.033	431.493
30	3.030	631.785
40	3.030	842.986
50	3.040	1052.479

Table 3: Linear regression data of the proposed HPLC method of Levetiracetam

Parameter	Method
Detection wavelength(λ max)	By UV at 243.2 nm
Linearity range (μ g/ml)	10-50 μ g/mL
Regression equation (Y=a+bx)	Y = 21.072X+0.5778
Slope(b)	21.072
Intercept(a)	0.5778
Standard deviation of slope (S _b)	0.7158
Standard deviation of intercept (S _a)	2.1670
Standard error of estimation (Se)	2.9944
Correlation coefficient (r ²)	0.9998
% Relative standard deviation*	0.1434
Percentage range of errors* (Confidence limits)	
0.05 significance level	0.36465
0.01 significance level	0.57232

*Average of 6 determinations; Acceptance criteria < 2.0.

Table 4: Specificity study

Name of the solution	Retention time (R _t) min.
Mobile phase	No peaks
Placebo	No peaks
Levetiracetam 10 μ g/mL	3.023min.

Table 5: Results of Precision study (Intra-day)

Sample	Concentration (µg/mL)	Injection no.	Peak area	%RSD (acceptance criteria < 2.0)
Levetiracetam	30	1	631.785	0.1434
		2	630.887	
		3	632.985	
		4	630.857	
		5	632.785	
		6	631.827	

Table 6: Results of Precision study (Inter-day)

Sample	Concentration (µg/mL)	Injection number	Peak area	%RSD (acceptance criteria < 2.0)
Levetiracetam	30	1	631.785	0.1443
		2	630.887	
		3	632.985	
		4	630.857	
		5	632.785	
		6	631.827	

Table 7: Recovery results of Levetiracetam

S. No.	Level of addition (%)	Amount added (µg/mL)	Amount recovered (µg/mL)	Mean % Recovery ± SD*	% RSD #
1	50 %	5	4.98	98.63 ± 0.305	0.265
		5	4.99		
		5	5.03		
2	100 %	10	9.98	99.63 ± 0.208	
		10	9.94		
		10	9.97		
3	150 %	15	14.98	99.72 ± 0.279	
		15	14.91		
		15	14.98		

#acceptance criteria < 2.0. * Mean of triplicate determination

Table 8: Robustness results of Levetiracetam

S. No	Parameter	Optimized	Used	Retention time (R _t), min	Plate count ^s	Peak asymmetry [#]	Remark
1.	Flow rate (±0.2 mL/min)	1.0 mL/min	0.8 mL/min	3.130	9510	1.060	*Robust
			1.0 mL/min	3.030	9503	1.063	*Robust
			1.2 mL/min	2.930	9458	1.046	*Robust
2.	Detection wavelength (±5 nm)	243.2 nm	238.2 nm	3.030	9504	1.063	Robust
			243.2 nm	3.030	9503	1.063	Robust
			248.2 nm	3.030	9503	1.063	Robust
3.	Mobile phase composition (±5 %)	50:50 v/v	55:45, v/v	3.028	9502	1.058	*Robust
			50:50, v/v	3.030	9503	1.063	*Robust
			45:55, v/v	3.033	9501	1.026	*Robust

Acceptance criteria (Limits) : # peak asymmetry <1.5, ^s Plate count >3000

Table 9: Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Parameter	Results
Limit of Detection (LOD)	0.468 µg/min
Limit of Quantitation (LOQ)	1.421 µg/min

Table 10: Assay results of Levetiracetam formulation

S.No	Formulations	Labelled amount	Amount found	% Assay ± RSD*
1	Keppra	500 mg	499.88 mg	99.97 ± 0.22
2	Epictal	500 mg	499.80 mg	99.96 ± 0.25

* Average of 6 determinations.

Figure 1: Chemical structure of Levetiracetam.

Figure 2: Calibration curve of Levetiracetam.

Figure 3: Chromatogram of placebo

Figure 4: Standard chromatogram of Levetiracetam (10 µg/mL)

Figure 5: Standard chromatogram of Levetiracetam (20 µg/mL)

Figure 6: Standard chromatogram of Levetiracetam (30 µg/mL)

Figure 7: Standard chromatogram of Levetiracetam (40 µg/mL)

Figure 8: Standard chromatogram of Levetiracetam (50 µg/mL)

Figure 9: A typical chromatogram of Levetiracetam standard

Figure 10: Chromatogram of market formulation (Keppra tablets) of Levetiracetam

RESULTS AND DISCUSSION

To optimize the HPLC parameters, several mobile phase compositions were tried. Various mobile phases having different ratios of methanol, acetonitrile and water were tried. Drug was retained in mobile phase consisting of methanol: water (60:40, v/v), Acetonitrile: water (80:20, v/v), Acetonitrile: water (70:30, v/v) tailing of the peak was observed. Excellent peak symmetry and satisfactory retention time was observed with the mobile phase consisting of 10 mM phosphate buffer (pH-3.0): acetonitrile (50:50, v/v at 1 mL/min flow rate). UV spectra of Levetiracetam showed that the drug absorbed maximum at 243.2 nm, so this wavelength was selected as the detection wavelength. Quantification was attained with Shimadzu SPD-20A Prominence UV-Vis detector at 243.2 nm based on peak area. The retention time for Levetiracetam was 3.030 minutes. System suitability parameters & optimized chromatographic conditions are shown in Table 1 and the calibration data are given in Table 2. The calibration curve for Levetiracetam was found to be linear over the range of 10-50 µg/mL. The data of regression analysis results are summarized in Table 3. So the linear regression data showed a good linear relationship over the concentration range of 10-50 µg/mL. The regression equation was found to be $Y = 21.072x + 0.5778$ with correlation coefficient is $r^2 = 0.9998$ which indicates this method has good linearity. The linearity of the graph is shown in Figure 2. The specificity was studied for the examination of the presence of interfering components, while the comparison of chromatograms there was no interference from placebo which is shown in Figure 3. They do not disturb the elution or quantification of Levetiracetam, furthermore the well-shaped peaks also indicate the specificity of the method. Therefore, it was concluded that the method is specific. The specificity results are summarized in Table 3. The representative standard chromatograms indicating the Levetiracetam are shown in Figure 4 to 8. The representative standard and sample chromatograms of Levetiracetam are shown in Figure 9 and 10 respectively. Precision was studied to find out intra and inter day variations in the test methods of Levetiracetam for the three times on the same day and different day. The % RSD for intraday and interday precision was found to be 0.1434 and 0.1443 respectively. The intra-day and inter-day precision obtained was % RSD (< 2.0) indicates that the proposed method is quite precise and results are shown in Tables 5 and 6. Repeatability of the method was studied by injecting 10 µg/mL solution of Levetiracetam for six times and peak area was measured and % RSD was calculated which was found to be 0.146 shows repeatability of the method. Accuracy of the method was evaluated by standard addition method in which appropriate portions of stock solutions of Levetiracetam were spiked into blank placebo matrix to produce concentrations of 50, 100 and 150% of the theoretical concentration. The mean recovery of spiked samples obtained was in range of 98.63-98.72% reveals no interference of excipients and shows that the method is accurate. The % recovery was found to be within the limits as listed in Table 7. Robustness was done by small changes in the chromatographic conditions like mobile

phase flow rate, wave length, mobile phase composition etc., It was observed that there were no marked changes in the chromatograms and RSD values for all these changes calculated were less than 2 indicate that proposed method is robust. In fact the parameters are within the limit which indicates that the method has robustness and suitable for routine use. The Robustness results are presented in Table 8. The limit of detection (LOD) and limit of quantitation (LOQ) was calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approximating the LOD and LOQ. The LOD and LOQ of the drug were found by scanning the solution of Levetiracetam having different lower concentrations. The limit of detection (LOD) was 0.468 µg/mL and the limit of quantitation (LOQ) was found to be 1.421 µg/mL which shows that this method is very sensitive. Results are summarized in Table 9. The proposed validated method was successfully applied to determine Levetiracetam in tablet form. The results obtained for tablets of Levetiracetam were comparable with the corresponding labelled amounts (500 mg/tab). Satisfactory results were obtained. The results were very close and in good agreement with the label value of commercial tablets. Finally the assay results of Levetiracetam formulation are tabulated in Table 10.

CONCLUSION

The present proposed developed RP - HPLC method for the quantification of Levetiracetam has several advantages like less retention time, excellent peak symmetry and good linearity, highly sensitive, simple, precise, accurate and robust and less retention time which were found with this analysis. This RP-HPLC method developed and fully validated for the quantitative determination of Levetiracetam in bulk and pharmaceutical tablet dosage forms. Statistical analysis of the results shows that the proposed procedure has good precision and accuracy. The method was completely validated shows satisfactory results for all the method validation parameters tested and method was free from interference of the other active ingredients and additives used in the formulation. In fact results of the study indicate that the developed method was found to be rapid, simple, reliable, accurate, linear, selective, sensitive, economical, reproducible and have short run time and only requires low cost technology which makes this method economically alternative for most clinical laboratories. Hence it can be concluded that this method may be employed for the routine quality control analysis of Levetiracetam in active pharmaceutical ingredient API and pharmaceutical preparations.

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