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A Novel Rapid Resolution High Performance Liquid Chromatographic Realated Substance Method Development and Validation of Levetiracetam in Bulk Drug Manufacturing of Active Pharmaceuticals Ingradient

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Abstract

Analytical method development is carried out to ensure that the API used and dosage forms that are developed and manufactured for human consumption are meeting the regulated quality norms & Validation of analytical method is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled as per ICH, USP, BP or any other suitable regulatory guidelines. The main study was depicts the development of a validated Rapid Resolution High Performance Liquid Chromatographic (RRHPLC) method for determination of Levetiracetam Related substances by Rapid Resolution High Performance Liquid Chromatographic (RRHPLC) in active pharmaceutical ingredient Bulk manufacturing. The Purity of Levetiracetam present in the active pharmaceuticals ingredient was found to be 99.87 %(99.8663). None of the impurities 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. The method validation was successfully applied for routine analysis for Bulk active pharmaceuticals ingredient levetiracetam samples. The developed Rapid Resolution Reverse phase liquid chromatography (RRRP-LC) method was validated with respect to System Suitability , linearity, , precision ,Range, Ruggedness, Test Solution and Mobile phase stability ,Robustness. The Present developed & validated method are run successfully for Levetiracetam samples in bulk drug active pharmaceutical ingredient manufacturing.

Key words: Levetiracetam, Rapid Resolution High Performance Liquid Chromatographic (RRHPLC), Determination, Validation, Pharmaceutical dosage form.

INTRODUCTION

Levetiracetam is a novel antiepileptic drug 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 conditions [4] such as Tourette syndrome, autism, and anxiety disorders. Levetiracetam seems to be a safe and effective treatment for migraine with aura. Chemically it is (αS)-α-ethyl-2-oxo-1-pyrrolidineacetamide) with a molecular formula of $C_8H_{14}N_2O_2$ 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 product. It is the S-enantiomer of etiracetam, structurally similar to the prototypical nootropic drug piracetam. Along with other anticonvulsants like gabapentin, it is also sometimes used to treat neuropathic pain. Levetiracetam has been approved in the European Union as a mono therapy treatment for epilepsy in the case of partial seizures, or as an adjunctive therapy for partial, myoclonic and tonic-clonic seizures. It is also used in veterinary medicine for similar purposes effects are behavioral and its benefit-risk ratio in these conditions is not well understood. Levetiracetam is, in general, well tolerated but may cause drowsiness, weakness, unsteady walking, coordination problems, headache, pain, forgetfulness, anxiety, irritability or hostility, dizziness, moodiness, nervousness, loss of appetite, vomiting, diarrhea, constipation, and changes in skin color. Some serious side effects can be depression, hallucinating (hearing voices or seeing visions that do not exist), suicidal thoughts, seizures that are worse or different, fever, sore throat, and other signs of infection, double vision, itching, rash, swelling of the face.A study published in 2005 suggests that the addition of pyridoxine (vitamin B6) may curtail some of the psychiatric symptoms. The structural formula of Levetiracetam is shown in Figure 1. [1-7]

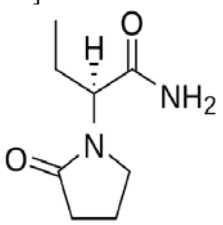

Fig. 1: Chemical structure of Levetiracetam.

Literature review reveals that few A novel Assay by RP-HPLC method for the analysis of levetiracetam in formulations and An improved Assay by RP-HPLC method for the quantitative determination and validation of levetiracetam in bulk and pharmaceutical formulation methods have been reported for analysis of levetiracetam .Although two LC simultaneous methods for pharmaceutical dosage and one for injections were reported in the literature review, existing methods were not subjected to Related substance by Rapid Resolution High Performance Liquid Chromatographic (RRHPLC) . More over reported methods were not much cost-effective in terms of solvent consumption and total run time of the analysis, so we decided to perform rapid, selective and precise indicating isocratic Related substance (or) Purity by Rapid Resolution High Performance Liquid Chromatographic (RRHPLC) method of levetiracetam in bulk drug manufacturing of active pharmaceuticals in gradient form which was not developed so far.

MATERIALS AND METHODS

Chemicals and Reagents:

Reference standard of Levetiracetam and samples, related Impurities (S)-2-Amino butyramide hydrochloride / (2-Amino Amide HCl) (MCL) and Levetiracetam , (S)-N-(1-Amino-1-oxobutan-2-yl)-4-chlorobutanamide(Impurity-1)and(S)-2-(2-Oxopyrrolidin-1-yl)butanoic acid (Impurity-2) was obtained from well reputed research laboratories and characterized by use of LCMS, NMR and IR . All the chemicals were analytical grade from Rankem Ltd., Mumbai, India, while acetonitrile (HPLC grade) Potassium dihydrogen phosphate , Sodium 1- Heptane sulphonate and Ortho phosphoric acid (HPLC grade) procured from Merck Pharmaceuticals Private Ltd., Mumbai, India and purchased from Merck Specialties Private Ltd., Mumbai, India. The Liquid Chromatography system was equipped with quaternary gradient pumps with auto sampler and column oven , auto injector connected to a variable wave length programmable ultra Violet visible detector all were controlled by Empower software and Manufactured by Agilent technologies with model .no: 1100 series.

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 Phosphate buffer (pH adjusted to 2.4 using dill. Ortho phosphoric acid) and acetonitrile in ratio of 92:8 v/v was selected as mobile phase Levetiracetam full fill all the criteria of system suitability. The mobile phase consisting of Phosphate buffer (pH adjusted to 2.4 using dill. Ortho phosphoric acid) and acetonitrile in ratio of 92:8 v/v was selected which gave sharp peak with retention time of 1.825 min. Similarly for the selection of diluent we tried the standard into different solvents like water, methanol, mobile phase and acetonitrile. Finally the selected diluent was Water .So finally the above said mobile phase and diluent was selected for analysis. Optimized chromatographic conditions are shown in Table 1.[9-10]

Table 1: Parameter Chromatographic conditions

Instrument	**Agilent High Pressure Liquid Chromatography Model -1100 series**
Column	Agilent ZORBAX SB C-18 column with 50 x 4.6mm internal diameter and 1.8µm particle size
Detector	Rapid resolution high performance liquid chromatographic UV detector (Flow cell volume is low)
Mobile phase	Mixture of Phosphate buffer (pH adjusted to 2.4 using dill.Ortho phosphoric acid) and acetonitrile in ratio of 92:8
Flow rate	1 mL/min.
Detection wave length By UV	at 200 nm.
Run time	15 Minutes
Temperature Ambient temperature	25oC
Volume of injection loop	5µL
Retention time (Rt)	1.825 minutes

Selection of detection wavelength

For the selection of analytical wavelength 3.0mg/ml Levetiracetam solution was prepared from standard drug solution and scanned in the range of 198 to 400 nm. From the UV spectra, the maximum λmax of Levetiracetam is found to be 200.2 nm. So this wavelength was selected as the detection wavelength for analysis. The selected mobile phase, diluent & wave length has given a sharp peak with low tailing factor 1.66 (<2) [9-10] .

Instrumentation and analytical chromatographic conditions :

The chromatographic analysis of method validation for related substance by Rapid resolution liquid chromatography determination of Levetiracetam was carried out on Agilent High Pressure Liquid Chromatography Model -1100 series containing quaternary pump, variable wave length programmable ultra violet visible detector and auto injector with up to 1µl-1000µl loop, column oven modules . Chromatographic analysis was performed using Agilent ZORBAX SB C-18 column with 50 x 4.6mm internal diameter and 1.8µm particle size. Metler electronic balance was used for weighing . The elution was carried out isocratically at flow rate of 1 ml/min using the mixture of Phosphate buffer (pH adjusted to 2.4 using dill.Ortho phosphoric acid) and acetonitrile in ratio of 92:8 v/v was selected as mobile phase and injection Volume 5 µl (µL –micro.litre) . The detection wavelength was set at 200 nm with a runtime of 15min . The mobile phase was prepared freshly and it was degassed by sonication for 5 min before use . The column was equilibrated for at least 10 min with the mobile phase flowing through the system. The column oven module and the High pressure liquid chromatography system were kept at 25ºC±2°C temperature and water is used as rinising solvent . [9-10].

Mobile phase preparation :

Accurately weighed 1.360 g of Potassium dihydrogen phosphate (KH2PO4) and 0.61 g of Sodium 1- Heptane sulphonate dissolved in 1000 ml of Mili-Q-water to get phosphate buffer. pH was adjusted to 2.4±0.05 with dilute ortho phosphoric acid . Above prepared buffer and acetonitrile were mixed in the proportion of 92:8 v/v. This mixture was sonicated for 10 minutes and filtered through 0.22 µm membrane filter and used as mobile phase.

Preparation of standard solutions & system suitability solution :

Pure standards of Levetiracetam were used as external standards in the analysis. Different concentrations of the standards were used based on the range required to plot a suitable calibration curve. About 30mg of the standard Levetiracetam was accurately weighed and transferred in to 10 ml volumetric flask and make up with sufficient diluent. Volumetric flask containing standard solution was sonicated for 10minutes. Similarly different concentrations of these standards were analysed using the same chromatographic conditions and a calibration curve was generated. The sample chromatogram and results recorded is in Fig. 2,Table2.

Diluent: Used diluent (Milliq -water) as blank.

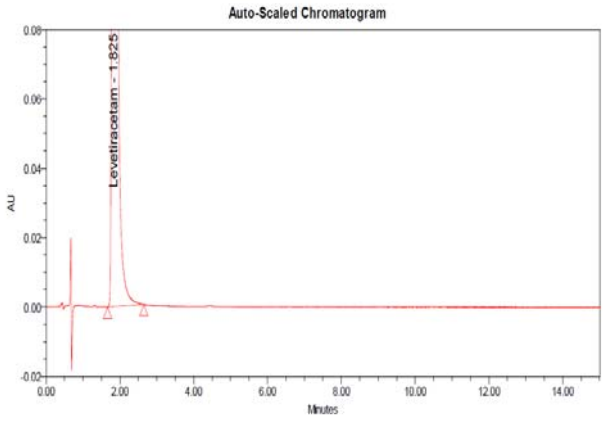

Fig. 2: Standard solution chromatogram

Table2: Standard system suitability parameters

Peak Results

	Name	RT	Area	Height	USP Plate Count	USP Resolution	USP Tailing
1	Levetiracetam	1.825	17594802	2903891	2651.19		1.66

System suitability solution : (Prepare fresh solution)

Weigh about 30.0 mg each of Levetiracetam standard, MCL , impurity-1 and impurity-2 in a 100 ml volumetric flask. Dissolve it by sonication till the solution is clear and make up to the mark with diluent. Pipette out 0.5 ml of the solution in to 100 ml volumetric flask and dilute up to the mark with diluent. System suitability solution were analysed using the same chromatographic conditions. The sample chromatogram and results recorded is in Fig. 3,Table 3.

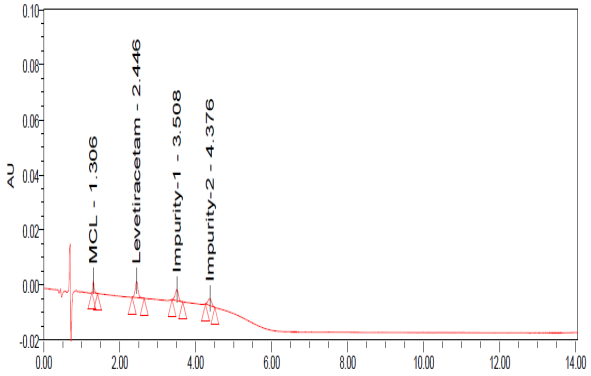

Fig. 3: System suitability solution chromatogram

Table3: System suitability solution parameters

Peak Results

	Name	Retention Time (min)	Area (µV*sec)	% Area	USP Resolution	USP Tailing	USP Plate Count
1	MCL	1.306	11024	13.3720		1.5	6384.43
2	Levetiracetam	2.446	30393	36.8647	11.44	1.2	5639.14
3	Impurity-1	3.508	24009	29.1217	7.19	1.0	7818.05
4	Impurity-2	4.376	17018	20.6416	4.98	1.0	9118.89

Preparation of Sample solutions:

Weigh accurately about 30.0 mg of Levetiracetam sample to be analysed in 10 ml volumetric flask. Dissolve with about 5 ml of diluent by sonication till the solution is clear and make up to the mark with diluent.. These solutions were analysed using the same chromatographic conditions. The sample chromatogram and results recorded is in Fig. 4,Table.4.

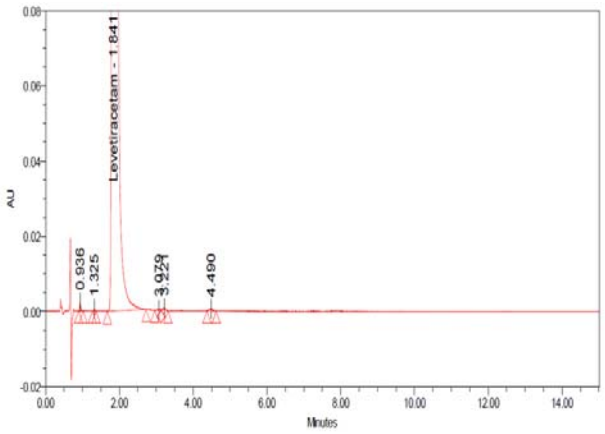

Fig. 4: Sample solution chromatogra

Table4: Sample solution system suitability parameters

Peak Results

	Name	RT	Area	Height	USP Plate Count	USP Resolution	USP Tailing
1		0.936	4659	2165	4765.31		1.41
2		1.325	1140	476	9345.35	6.46	1.00
3	Levetiracetam	1.841	17088434	2898596	2803.63	5.04	1.61
4		3.079	1212	202			
5		3.221	3852	679	7971.65		
6		4.490	3785	562	9805.02	6.95	0.96
None							

METHOD VALIDATION PROCEDURE

After the completion of High pressure liquid chromatography method development, the objective of the method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in International Conference on Harmonisation (ICH) guidelines. The method was validated for system suitability, System precision, specificity, linearity, limit of detection and limit of quantification, Ruggedness & Range, Method precision, Robustness.[11-14]

System suitability parameter :

To verify that analytical system is working properly and can give accurate and precise results, the system suitability parameters are to be set. System suitability tests were carried out on freshly prepared 30mg standard solutions of Levetiracetam and MCL , impurity-1 and impurity-2 in a 100 ml volumetric flask. Dissolve it by sonication till the solution is clear and make up to the

mark with diluent. Pipette out 0.5 ml of the solution in to 100 ml volumetric flask and dilute up to the mark with diluent. System suitability solution were analysed using the same chromatographic conditions. it was calculated by determining the standard deviation of the values were recorded in Table 5. The system suitability method acceptance criteria set in each validation run were tailing factor ≤2.0 and theoretical plates >2000, Resolution >1.5 between two closely eluting peaks (Half width) or product specific.In all cases, the relative standard deviation (R.S.D) for the analytic peak area for two consecutive injections was not More than 2%.A chromatogram obtained from reference substance solution is presented. System suitability parameters were shown in Table.5. System suitability solution Standard chromatogram was given in Figure.5. The total results of system suitability studies summarized in Table 6. In this studies %RSD value of retention times, peak areas, tailing factor and theoretical plate count, Resolution were found to be less than 2% for (S)-2-Amino butyramide hydrochloride / (2-Amino Amide HCl) (MCL) and Levetiracetam , (S)-N-(1-Amino-1-oxobutan-2-yl)-4-chlorobutanamide(Impurity-1)and(S)-2-(2-Oxopyrrolidin-1-yl)butanoic acid (Impurity-2). .[11-14]

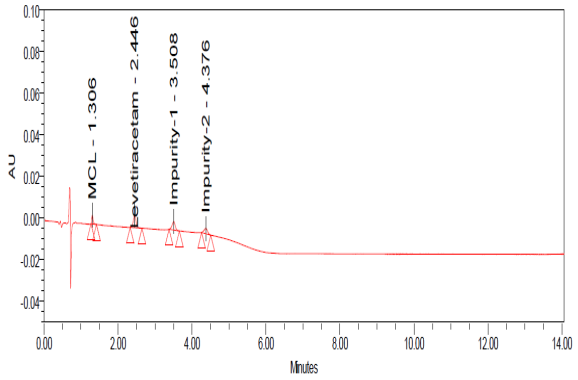

Fig. 5: System suitability solution chromatogram

Table5: System suitability solution parameters

Peak Results

	Name	Retention Time (min)	Area (µV*sec)	% Area	USP Resolution	USP Tailing	USP Plate Count
1	MCL	1.306	11024	13.3720		1.5	6384.43
2	Levetiracetam	2.446	30393	36.8647	11.44	1.2	5639.14
3	Impurity-1	3.508	24009	29.1217	7.19	1.0	7818.05
4	Impurity-2	4.376	17018	20.6416	4.98	1.0	9118.89

Results:System suitability parameter:

Table-6: Results for System suitability parameter

S.no	Name of the Impurity	Retention Time	Area	Resolution	Tailing	Plate count
1	MCL	1.306	11024	NA	1.5	6384.43
		1.305	11005	NA	1.5	6384.57
		1.304	11015	NA	1.5	6373.15
		1.305	11037	NA	1.5	6384.73
		1.304	11047	NA	1.5	6354.63
		1.305	11057	NA	1.5	6383.15
	%RSD	0.041	0.18	NA	0	0.19
2	Levtiracetam	2.446	30393	11.44	1.2	5639.14
		2.445	30333	11.42	1.2	5635.15
		2.443	30298	11.34	1.2	5625.14
		2.442	30253	11.54	1.2	5624.14
		2.440	30278	11.68	1.2	5680.15
		2.441	30268	11.50	1.2	5674.18
	%RSD	0.09	0.17	0.86	0	0.44
3	Impurity-1	3.508	24009	7.19	1.0	7818.05
		3.507	23119	7.15	1.0	7817.49
		3.506	24120	7.13	1.0	7801.01
		3.505	23919	7.14	1.0	7800.25
		3.502	23818	7.18	1.0	7799.25
		3.501	23578	7.16	1.0	7812.56
	%RSD	0.08	1.53	0.32	0	0.11
4	Impurity-2	4.376	17018	4.98	1.0	9118.89
		4.271	17045	4.88	1.0	9128.39
		4.175	17055	4.99	1.0	9138.47
		4.389	16999	5.01	1.0	9108.49
		4.400	17001	5.02	1.0	9129.99
		4.355	16988	4.91	1.0	9109.87
	%RSD	0.64	0.16	1.14	0	0.13

System precision:

The system precision is checked by using standard chemical substance to ensure that the analytical system is working properly. The retention time and area of 10 determinations is measured and % RSD shall be calculated and it is Not More than 1.0%. The total results of system precision studies summarized in Table 7. .[11-14]

Preparation of standard solution:

Accurately weigh &transfer about 30.0mg of Levetiracetam standard into a 10ml volumetric flask. Dissolve dilute to volume with diluent.

Results:System precision:

Table-7: System Precision parameters

Preparation	Area	Retention time	Tailing Factor
1	17594802	1.82	1.66
2	17617838	1.83	1.60
3	17693702	1.84	1.61
4	17670882	1.82	1.64
5	17658175	1.83	1.63
6	17691672	1.83	1.64
7	17650660	1.83	1.61
8	17658918	1.83	1.61
9	17626661	1.82	1.61
10	17651203	1.83	1.67
Average	17651203	1.83	NA
STDEV	31332.62	0.004	NA
%RSD	0.18	0.24	Tailing<2.

Requirement	Result	Acceptance Criteria
Relative Standard deviation (RSD) for 10injections	0.18	Not More than 1.0%

From the above tabulated data, it can be concluded that the system precision parameters meets the requirements of method validation.

Specificity Parameter:

Specificity is the ability of analytical method to assess the analyte in the presence of components that may be expects to be present, such as impurities, degradation products and matrix components. .[11-14]

Specificity tests were carried out on above prepared standard solution of Levetiracetam and it was determining by injecting the mobile phase and placebo solution in triplicate and recording the chromatograms specify solution (standard solution) for Levetiracetam Standard Solution.

Table8: Specificity Parameters

Peak name	RT
Mobile phase	No peaks
Placebo	No peaks
Levetiracetam standard solution	1.825

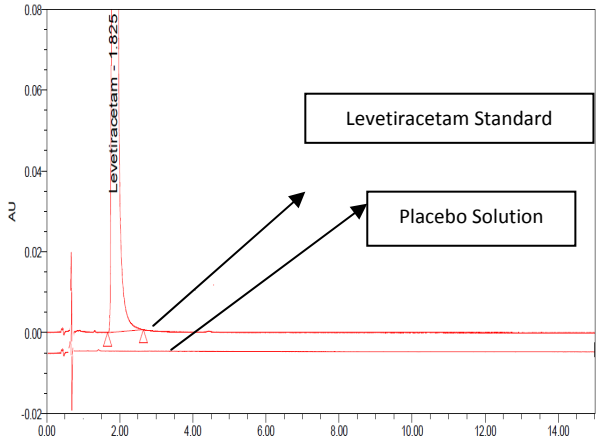

Fig. 8: Over laid chromatogram for specificity.

From the above data (Table 8),(**Fig. 8)** Proves that method is specific that is there is no interference of placebo peaks in Levetiracetam Standard solution.

Linearity : The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in sample within a given range . .[11-14]

The developed method has been validated as per International Conference on Harmonisation (ICH) guidelines the Standard test solutions of Levetiracetam in the mass concentration range of 25% to 125% was injected into the chromatographic system. The chromatograms were developed and the peak area was determined for each concentration of the drug solution. Calibration curve of Levetiracetam was obtained by plotting the peak area ratio versus the applied concentrations of Levetiracetam. The linear correlation coefficient was found to be 1.0 (0.999).

The Values & Calibration curve were recorded in Table 10 & Fig. 9.

Linearity was demonstrated by injecting test solution from,**25%,50%,75%,100%,125%**and **150%** to the desired test concentration (i.e.30mg/10ml) .

Preparation of linearity solutions for test solution:

Table 9: Linearity different levels of concentrations

Level	Weight of test solution taken	Dissolved and made up volume with diluent
25%of test concentration	7.552mg	10ml
50%of test concentration	15.030mg	10ml
75%of test concentration	22.663mg	10ml
100%of test concentration	30.190mg	10ml
125%of test concentration	37.598mg	10ml
150%of test concentration	45.347 mg	10ml

Injected each solution once into the HPLC system and plotted the calibration curve by taking concentration (mg/ml) on X-axis and peak area on Y-Axis and calculated the correlation coefficient .

Table 10: Linearity parameters

Level	Concentration in mg/ml	Area
25%	0.7552	6018929
50%	1.5030	9243090
75%	2.2663	12654693
100%	3.0190	15448038
125%	3.7598	18650158
150%	4.5347	21241671
Slope		122432.296
Correlation Co-efficient		0.99922
Regression Coefficient		0.998438

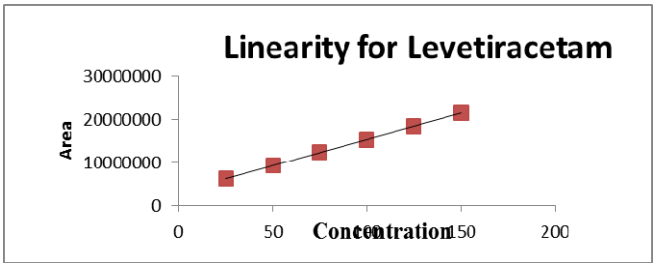

Fig. 9: Calibration curve for Linearity

Table 11: Residual output for Linearity parameters

RESIDUAL OUTPUT		
Observation	Predicted Y	Residuals
1	6224078	-205149
2	9284885.4	-41795.4
3	12345692.8	309000.2
4	15406500.2	41537.8
5	18467307.6	182850.4
6	21528115	-286444

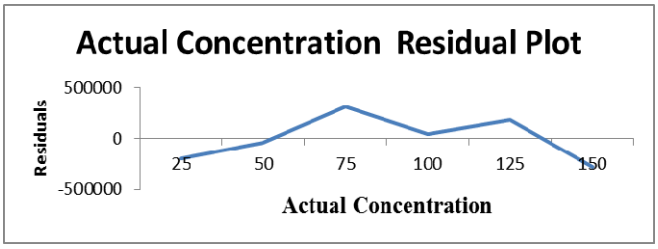

Fig. 10: Residual plot for linearity parameters

From the data, it is clear that the area response of Levetiracetam vs concentration in percentage of Levetiracetam linear in the range of interest. The correlation coefficient and regression coefficient calculated from regular plot is greater than 0.999. Hence the method is linear for the residual determination of Levetiracetam.

Limit of Detection & Limit of Quantification:

Limit of detection is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. .[11-14]

Limit of quantification is the lowest amount of analyte in a sample that can be quantitated with acceptable precision, under the stated experimental conditions. .[11-14]

Limit of detection and Limit of quantitation were calculated using following formula.

LOD = (3.3 X Residual standard deviation) / slope.

LOQ = (10X Residual standard deviation) / slope .

The LOD and LOQ values are presented in Table 12.

Table 12: LOD&LOQ Theoretical Results

Levetiracetam	
Theoretical LOD in mg/ml	0.61mg/ml
Theoretical LOQ in mg/ml	1.85mg/ml

Performed a regression analysis of the linearity data with concentration vs ppm on X-axis. Calculated the residual standard deviation of the Y data. Calculated the slope of the linearity curve generated with concentration on X-axis and area response on Y-axis**.** The % RSD for area response of Levetiracetam six replicates at LOQ level was found to be 0.10%.

METHOD PRECISION

Precision is a measure of the degree of repeatability of the analytical method , determined by analyzing sufficient number of aliquots of a homogenous sample solution. To study precision, six replicate test solutions of 100% and 150% Levetiracetam were prepared and analyzed using the proposed method i.e Injected each solution once in to the chromatograph. Calculated %RSD for area. The percentage of relative standard deviation (% RSD) for peak responses was calculated and it was found to be 0.18 & 0.42% which is well within the acceptance criteria of not more than 2.0% and % of RSD for purity at 100% of specification and 150% of specification level was calculated and it was found to be 0.01 & 0.00% which is well within the acceptance criteria of not more than 2.0% Results of Method precision studies are shown in Table.15& 16. .[11-14]

Preparation of Method precision solutions for test solution:

Preparation of 100% test solution:

Table 13: Preparations of 100% test solution

Spiked Prep.	Weight taken	Dissolved in diluents
1	30.19mg	10ml
2	30.41mg	10ml
3	30.56mg	10ml
4	30.58mg	10ml
5	30.59mg	10ml
6	30.66mg	10ml

Preparation of 150% test solution:

Table 14: Preparations of 150% test solution

Spiked Prep.	Weight taken	Dissolved in diluents
1	45.01mg	10ml
2	44.95mg	10ml
3	45.10mg	10ml
4	46.16mg	10ml
5	44.98mg	10ml
6	45.04mg	10ml

Results of Method precision :

Table 15: Method precision Results For Peak Responses of 100% and 150% of specification level

Preparation	100%	150%
1	17065832	20950692
2	16982901	21077153
3	17023662	21161669
4	17042603	21172824
5	17022830	21089821
6	17051737	21176365
Average	17033723	21106988
STDEV	31178.9	89208.3
% RSD	0.18	0.42
Acceptance criteria	The %RSD for impurity area-**Not more than 2%**	
Status	Meet the criteria	

Table 16: Method precision Results for purity at 100% and 150% of specification level

Preparation	100%	150%
1	99.8663	99.8387
2	99.8657	99.8429
3	99.8495	99.8351
4	99.8633	99.8364
5	99.8513	99.8300
6	99.8753	99.8364
Average	99.8619	99.8366
STDEV	0.010	0.004
% RSD	0.01	0.00
Acceptance criteria	The %RSD for impurity area-**Not more than 2%**	
Status	Meet the criteria	

Range: Range is defined as the range of concentration in which method is linear, precise and accurate .For range,data was considered from linearity and precise sections. Range was performed for the test solution at 25% to 150% of specification level and found it to be precise , accurate and linear. .[11-14].

Linearity results:

Table 17:Linearity Results for Range

Linearity	Correlation:0.9992
Acceptance criteria	Correlation Coefficient-**Not less than 0.999**

Precision results:

Table 18: Precision Results for Range

Precision(%RSD)	
At 100% level	0.18%
At 150% level	0.42%
Acceptance criteria	The % RSD for impurity content-**Not more than than 2.0%**

RUGGEDNESS (OR) INTERMEDIATE PRECISION:

Ruggedness (or) Intermediate precision was demonstrated by determing the precision of method by analyzing same sample on different system , by different analyst and in different column. To study Ruggedness , we conducted a two studies as Study-1 & study-2 details are enclosed in Table: 15 and six replicate test solutions of 100% of

specification level test solutions were prepared and analyzed same sample on different system , by different analyst and in different column using the proposed method i.e Injected each solution once in to the chromatograph. Calculated %RSD for area & purity . .[11-14].

The percent relative standard deviation (% RSD) for area & Purity of Study-1**(1st System,1st Column and 1stAnalyst)** was calculated and it was found to be 0.18 & 0.01% which is well within the acceptance criteria of not more than 2.0% and using same analyst ,same system with different column %RSD for area & purity at 100% of specification level was calculated and it was found to be 0.18 & 0.01% which is well within the acceptance criteria of not more than 2.0% Results of Ruggedness studies are shown in Table.21 & 22.

Simillarly for The percent relative standard deviation (% RSD) for area & Purity of Study-2**(1st System, 2nd Column and 2nd Analyst)** was calculated and it was found to be 0.18 & 0.01% which is well within the acceptance criteria of not more than 2.0% and using same analyst ,same system with different column %RSD for area & purity at 100% of specification level was calculated and it was found to be 0.18 & 0.01% which is well within the acceptance criteria of not more than 2.0% Results of Ruggedness studies are shown in Table.19 & 20.

Table 19: System, Column and analyst details

Study	System	Analyst	Column	Day
1	QC-130	I	G-09/05	1
2		II	G-09/07	2

Table 20: Preparation of 100% of specification level test solutions for Study-1(1stAnalyst)

Preparation	Weight taken	Dissolved in diluents
1	30.19mg	10ml
2	30.41mg	10ml
3	30.56mg	10ml
4	30.58mg	10ml
5	30.59mg	10ml
6	30.66mg	10ml

Table 21: Are and Purity Results for Study-1 Test solution 100% of specification Level (1st System,1st Column and 1stAnalyst)

Preparation	Area	Purity
1	17065832	99.8663
2	16982901	99.8657
3	17023662	99.8495
4	17042603	99.8633
5	17022830	99.8513
6	17051737	99.8753
Average	17033723	99.8619
STDEV	31178.9	0.010
% RSD	0.18	0.01
Acceptance criteria	The %RSD for impurity area & Purity **-Not more than 2.0%**	

Table 22: Are and Purity Results for Study-1 Test solution 100% of specification Level (1st System, 2nd Column and 1stAnalyst)

Preparation	Area	Purity
1	17065832	99.8663
2	16982901	99.8657
3	17023662	99.8495
4	17042603	99.8633
5	17022830	99.8513
6	17051737	99.8753
Average	17033723	99.8619
STDEV	31178.9	0.010
% RSD	0.18	0.01
Acceptance criteria	The %RSD for impurity area & Purity **-Not more than 2.0%**	

Table 23: Preparation of 100% of specification level test solutions for Study-1(2nd Analyst):

Preparation	Weight taken	Dissolved in diluent
1	30.23mg	10ml
2	30.03mg	10ml
3	30.64mg	10ml
4	30.19mg	10ml
5	30.35mg	10ml
6	30.12mg	10ml

Table 24: Are and Purity Results for Study-1 Test solution 100% of specification Level (2nd Analyst):

Preparation	Area	Purity
1	16000265	99.9067
2	16035597	99.9091
3	16000838	99.9169
4	16082211	99.9070
5	16099078	99.9079
6	16138684	99.9076
Average	16059446	99.9092
STDEV	56344.706	0.004
% RSD	0.35	0.00
Acceptance criteria	The %RSD for impurity area & Purity **-Not more than 2.0%**	

TEST SOLUTION AND MOBILE PHASE STABILITY: Established the stability of standard solution ,test solution and mobile phase which was used in estimation of % of purity, over a period of 2 days. Prepared the standard solution and test solution at 100% of specification. Prepared the mobile phase as per the test method and kept it well-closed condition. Injected blank, Standard solution and test solution freshly and injected into HPLC system by following the conditions described in test method. Calculated the % purity for test solution as per the test method. Stored the mobile phase on bench top .Stored the standard solution and test sample solution on bench top. .[11-14]

On day 1 and day 2, used the stored mobile phase and injected stored system suitability solution and test solutions followed by injected freshly prepared standard solution and test solution. Test solution and mobile phase are found to

be stable for 48hours from the time of preparation .System suitability results of resolution solution are within the acceptance criteria up to 48hours from the time of preparation.

The results from these studies indicated, the standard & sample solution was stable at room temperature for at least 48(48h). Calculated the % of purity for stored test solutions and freshly prepared solutions as per the test method for estimation of test sample solution and mobile phase stability. Results of test solution and mobile phase stability are shown in Table.25 & 26 .The sampled chromatograms are recorded as below in Fig.11, Fig.12, Fig.13, Fig.14, Fig.15, Fig.16, Fig.17, Fig.18 & Fig.19

Solution Stability results & Mobile Phase Stability results :

Table 25: Solution Stability result

Impurity	Solution Stability			Variation		%Variation		Acceptance Criteria
	Initial	1st day	2nd day	1st day	2nd day	1st day	2nd day	
Purity	99.8597	99.8554	99.8138	0.0043	0.0459	0.00004	0.0005	Within±15%

Table 26: Mobile Phase Stability results

Impurity	Solution Stability			Variation		%Variation		Acceptance Criteria
	Initial	1st day	2nd day	1st day	2nd day	1st day	2nd day	
Purity	99.8597	99.8918	99.8593	0.0321	0.0004	0.0003	Not considerable	Within±15%

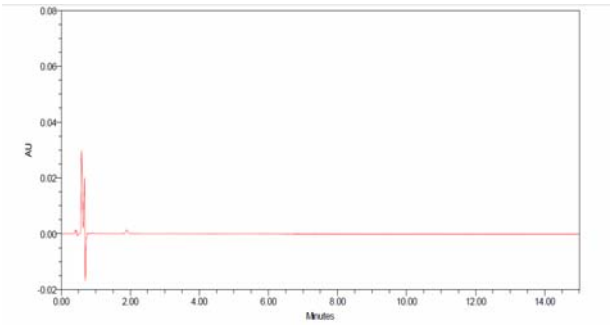

Fig. 11: Blank Solution Initially prepared

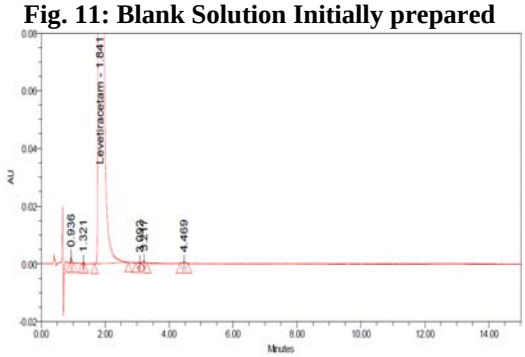

Fig. 12: Standard Solution Initially Prepared

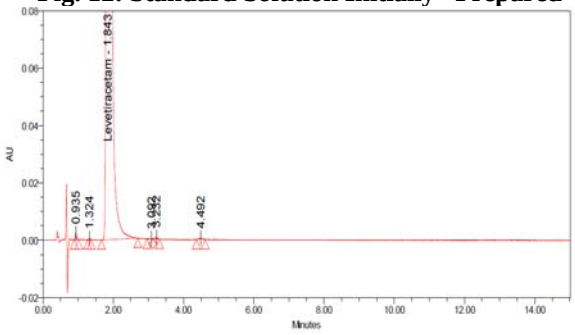

Fig. 13: Sample Solution Initially Prepared

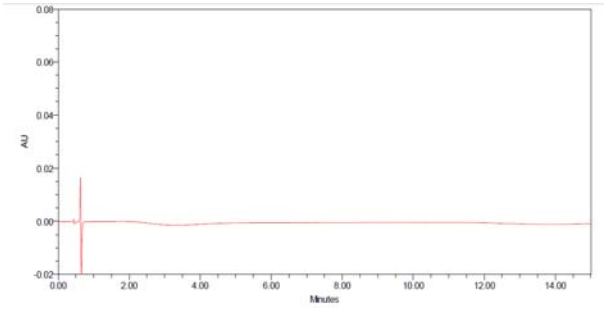

Fig. 14: Blank After 24 hours injected chromatogram

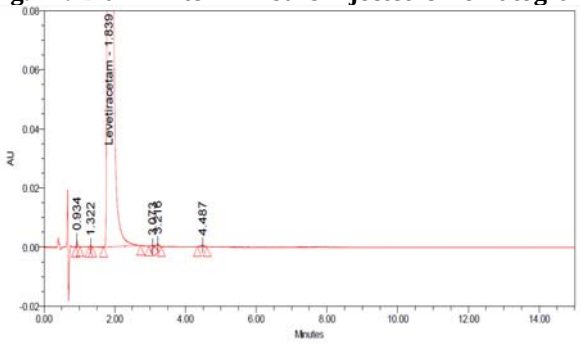

Fig. 15:Standard After 24hours

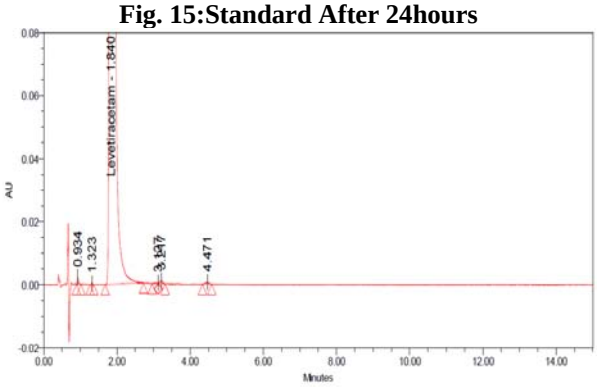

Fig. 16: Sample After 24hours

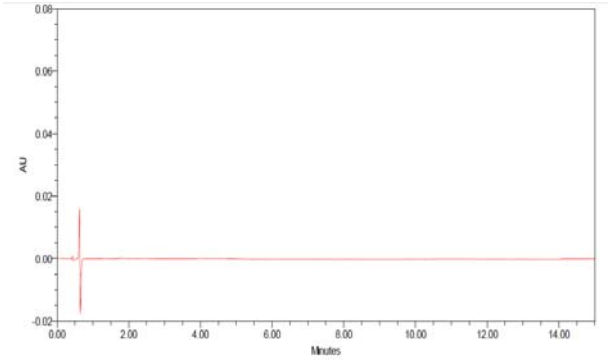

Fig. 17: Blank After 48 hours injected chromatogram

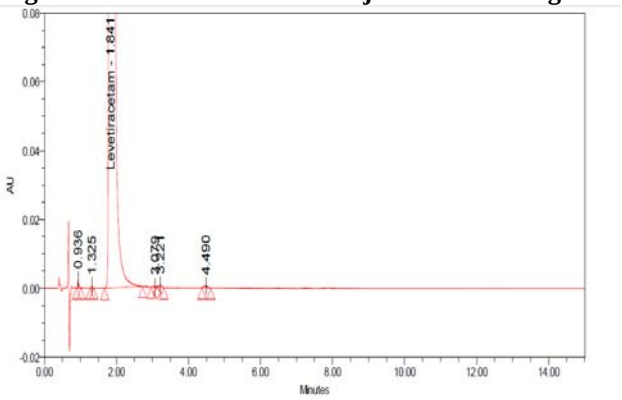

Fig. 18: Standard After 48hours

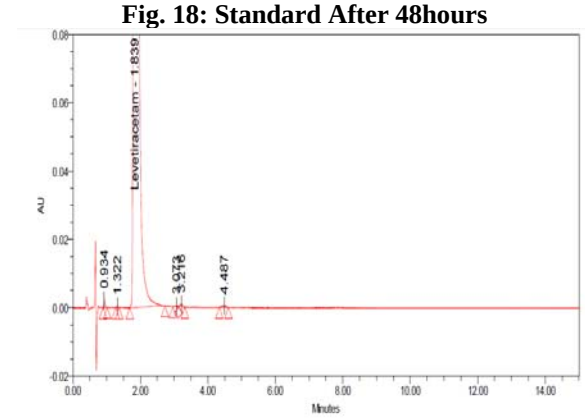

Fig. 19: Sample After 48hours

ROBUSTNESS:

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage and it was evaluated by the analysis of Levetiracetam under different experimental conditions such as making small changes in flow rate (± 0.2 mL/min), Column oven temperature (± 5°C), and pH (± 0.2). The results are presented in Table 8. .[11-14].

VARIATION IN FLOW RATE:

Adjusted the mobile phase flow rate as per the following in Table 23 .as two conditions separately while other method parameters remain unchanged. Prepared test solution at 100% specification as per test method .Injected blank, test solution once at each of the above mentioned conditions into the chromatograph and evaluated. Results for Variation in flow rate in Table 27.

Table 27: Variation in flow rate

Parameter	As Such	Condition-1	Condition-2
Flow rate	1.0ml/min.	0.8ml/min.	1.2ml/min.

Table 28: Results for Variation in flow rate of Levetiracetam

Impurity name	As Such	Condition-1	Condition-2
Principal peak of Levetiracetam	1.83RT	2.09RT	1.40RT

VARIATION IN COLUMN OVEN TEMPERATURE:

Adjusted the column oven temperature as per the following in table.25 as two conditions separately while other method parameters remain unchanged. Prepared test solution at 100% specification as per test method .Injected blank, test solution once at each of the above mentioned conditions into the chromatograph and evaluated. Results for Variation in column oven temperature in Table 29.

Table 29: Variation in column oven temperature

Parameter	As Such	Condition-1	Condition-2
Column oven temperature	25°C	20°C	30°C

Table 30: Results for Variation in column oven temperature

Impurity name	As Such	Condition-1	Condition-2
Principal peak of Levetiracetam	1.83RT	1.71RT	1.65RT

VARIATION IN pH OF MOBILE PHASE:

Adjusted the pH in mobile phase as per the following table.27 as two conditions separately while other method parameters remain unchanged. Prepared test solution at 100% specification as per test method .Injected blank, test solution once at each of the above mentioned conditions into the chromatograph and evaluated. Results for Variation in column oven temperature in Table 31.

Table 31: Variation In pH Of Mobile Phase

Parameter	As Such	Condition-1	Condition-2
pH	2.4	2.2	2.6

Table 32: Results for Variation In pH Of Mobile Phase

Impurity name	As Such	Condition-1	Condition-2
Principal peak of Lev	1.83RT	1.86RT	1.77RT

Record of analysis for levetiracetam samples:

Triplicate levetiracetam samples are run successfully by using this method and the experimental results & chromatograms are recorded in (Fig. 20), (Fig. 21), (Fig. 22), (Fig. 23) and (Fig. 24).

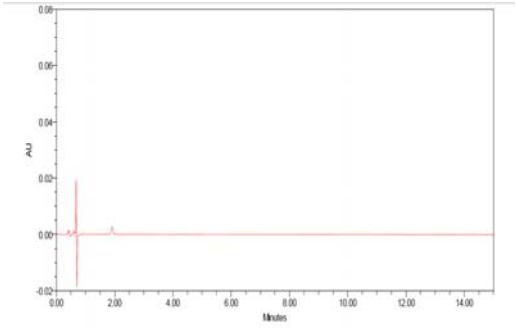

Fig. 20: Blank

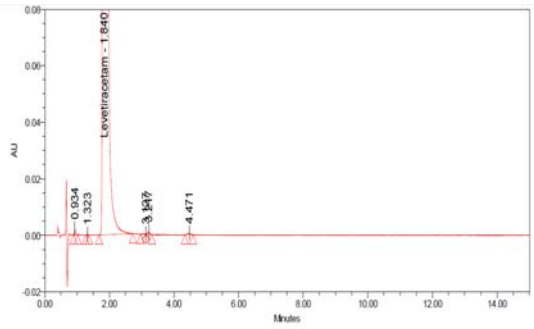

Fig. 21: Standard chromatogram

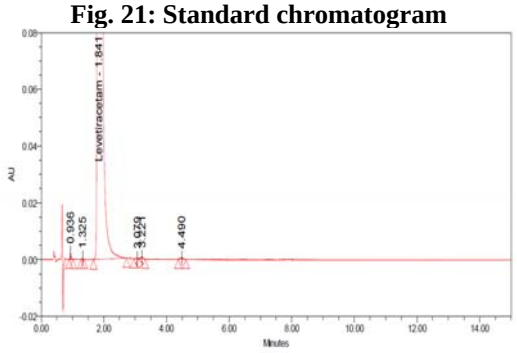

Fig. 22: 1st Sample chromatogram

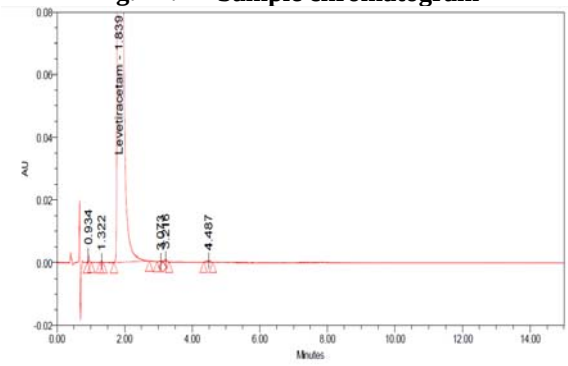

Fig. 23: 2nd Sample chromatogram

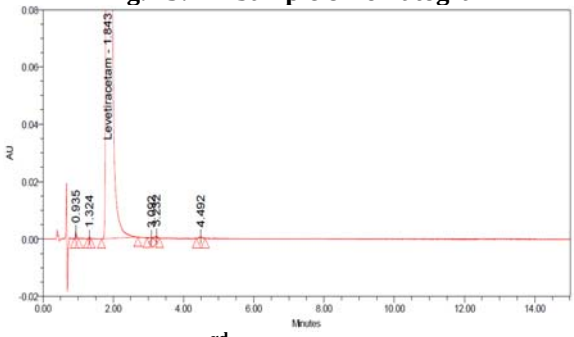

Fig. 24: 3rd Sample chromatogram

Significance of the developed method

Developed isocratic Rapid Resolution High Performance Liquid Chromatographic (RRHPLC) indicating method has many advantages over reported methods: (a) the method was simple because mobile phase used was cheap and easily available; (b) total run of chromatogram was 15min and Levetiracetam ,MCL,Impurity-1 & Impurity-2 were eluted within 4.5 min indicating that very less amount of mobile phase was consumed. (c) the limit of detection for Levetiracetam was 0.61mg/ml and the limit of quantification was1.85mg/ml, respectively, indicating that the method was sensitive and rapid; (d) specificity study(Figs. 1 and 2) and indicate that the method was very specific, stable in the proposed method.(e) mode of separation is isocratic which mean sit is easy to operate throughout the process with out any complications (f) simultaneous estimation of different brands gives precise results indicating that developed method is compatible to estimate in different active pharmaceuticals ingradient (g) validation of the developed method as per ICH guideline indicates that the method was highly precise, rapid, simple, economical, sensitive accurate, robust and specific for determination of related impurities of Levetiracetam in bulk and pharmaceutical dosage form.

RESULTS

As there is a growing demand of Levetiracetam in active pharmaceutical ingredient market, it is required to develop fast, cost-effective, stable, precise and sensitive analytical method .The primary target in developing & Validate this Rapid Resolution High Performance Liquid Chromatographic (RRHPLC) method is to achieve the optimum resolution between products with other products to supply high purity of drug i.e. Based on the above observed results the developed Rapid Resolution High Performance Liquid Chromatographic (RRHPLC) validation method for Levetiracetam is valid and run successfully the summary and evaluation of results are in below (Table 11)

Table 11:Summary and Evaluation of Results

Validation parameter	Acceptance criteria	Results
System suitability	The system suitability method acceptance criteria set in each validation run were tailing factor ≤2.0 and theoretical plates >2000, Resolution >1.5 between two closely eluting peaks (Half width) or product specific.In all cases, the relative standard deviation (R.S.D) for the analytic peak area for two consecutive injections was not More than 2%.	System suitability parameter meets the criteria and %RSD value of retention times, peak areas, tailing factor and theoretical plate count, Resolution were found to be less than 2%.
System precision	The % RSD of retention time and area of 10 determinations should not more than 1.0%	System precision parameters meets the requirements of method validation.
Specificity	No interference of placebo peaks in Levetiracetam Standard solution	Method is specific that there is no interference of placebo peaks in Levetiracetam Standard solution
Linearity	The correlation coefficient and the regression coefficient between concentration and area	The correlation coefficient and regression coefficient calculated from regular plot is greater than 0.999

	response of Levetiracetam should be NLT 0.995	
LOD/LOQ	The% of RSD for area response of Levetiracetam from six replicates at LOQ level should be NMT 10.0%	LOD/LOQ parameters meets the requirements of method validation.
Method Precision	% of RSD for purity and Area at 100% of specification and 150% of specification levet not more than 2.0%	Method precision parameter meets the criteria and %RSD value of retention times, peak areas were found to be less than 2%.
Range	At 25% to 150% of specification level to be precise , accurate and linear.	Meet the criteria At 25% to 150% of specification level and found it to be precise , accurate and linear.
Ruggedness (or) Intermediate Precision:	Different system , by different analyst and in different column the %RSD for area & purity at 100% of specification level was not more than 2.0%.	%RSD for area & purity at 100% of specification level was calculated which is well within the acceptance criteria of not more than 2.0%
Test solution and Mobile phase Stability	Established the stability of standard solution ,test solution and mobile phase which was used in estimation of % of purity, over a period of 2 days.	The results from these studies indicated, the standard & sample solution , Mobile phase was stable at room temperature for at least 48(48h).
Robustness	A small deliberate variations in method parameters like flow rate (± 0.2 mL/min), Column oven temperature (± 5°C), and pH (± 0.2).	From the results reveal that the method is robust.

CONCLUSION

The Isocratic Rapid Resolution High Performance Liquid Chromatographic (RRHPLC) method developed for the analysis of levetiracetam in their pharmaceutical preparations is precise, accurate, and with a reasonable run time. The developed method was validated as per ICH Guidelines shows that the developed method was highly specific and robust so that it can be effec-tively applied for routine analysis in research institutions, in quality control department of pharma-ceutical industries, and in approved testing laboratories, from the above experimental data on the various method validation parameters, it is proved that this method which was designed to determine the related impurities in Levetiracetam is precise, accurate, linear, rugged, robust and range from 25% to 150% of the Specification . Hence, the method can be used for routine application.

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