International Journal of Research in Pharmacy and Science

Available online at www.ijrpsonline.com

Research Article

An improved RP-HPLC method for the quantitative determination and validation of Retigabine in bulk and Pharmaceutical formulation

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Received: 09-10-2014
Review completed: 04-12-2014
Accepted: 29-12-2014

Access this article online

QR Code

Website: www.ijrpsonline.com

ABSTRACT

For the first time a convenient, simple, specific, accurate, precise, rapid, inexpensive isocratic Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the quantitative determination of Retigabine in pharmaceutical tablet dosage form. RP-HPLC method was developed by utilizing Welchrom C_{18} Column (4.6 X 250 mm, 5µm), 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^{-1} with the responses measured at 302.8 nm using Shimadzu SPD-20A Prominence UV-Visible detector. The retention time of Retigabine was found to be 5.670 minutes. Linearity was established for Retigabine in the range of 2-10 µg.mL^{-1} with correlation coefficient 0.9997. The LOD and the LOQ were found to be 0.1658 µg.mL^{-1} and 0.5025 µg.mL^{-1} respectively. The amount of Retigabine present in the formulation was found to be 99.90 %. The validation of the developed method was carried out for specificity, linearity, precision, accuracy, robustness, limit of detection, limit of quantitation. The developed method can be utilized for regular quality control analysis of Retigabine in pharmaceutical dosage form.

Key words: Retigabine, RP-HPLC, Determination, Validation, Pharmaceutical dosage form

INTRODUCTION

Retigabine (Ezoganine) is a novel anticonvulsant drug utilized for the treatment of partial epilepsies[1-3]. It is chemically N-[2-Amino-4-(4-fluorophenyl-methylamino)-phenyl] carbamic acid ethyl ester. Retigabine works primarily as a potassium channel opener[4-10] that is, by activating a certain family of voltage-gated potassium channels in the brain. Retigabine pharmacokinetics is primarily linear over the single dosage range of 25 mg to 600 mg in healthy human volunteers[11]. This mechanism of action is unique among antiepileptic drugs, and may hold promise for the treatment of other neurological conditions, including migraine and neuropathic pain. For the synthesis of Retigabine[12] starting materials are 2-nitro-1, 4-phynylene diamine is reacted with 4-fluoro benzaldehyde. It is easily synthesized in 3 step .procedure. Retigabine is rapidly absorbed and distributed in the man with oral bioavailability[13] of 60% and plasma binding of the drug is approximately 80%. It is purple coloured compound with molecular weight of 376.23, log p of 2.0 and pKa of 10.8. The chemical structure of Retigabine is shown in the following Figure 1.

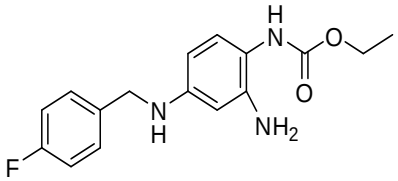

Figure 1 : Chemical structure of Retigabine

A detailed literature survey reveals that no RP-HPLC method has been reported for the determination of Retigabine in pharmaceutical dosage form hitherto. Hence the present method was developed to quantify Retigabine in tablet dosage form by isocratic RP-HPLC. Each tablet

contains 50 mg, 100 mg, 200 mg, 300 mg, and 400 mg of Retigabine. The drug was developed by Valeant Pharmaceuticals[14] and GlaxoSmithKline.

MATERIALS AND METHODS

Chemicals and Reagents:
The reference sample of Retigabine standard was kindly supplied as gift sample by Hetero Drugs Ltd., Hyderabad, and Andhra Pradesh, India. All the chemicals were analytical grade. Potassium dihydrogen orthophosphate and phosphoric acid from Rankem Ltd., Mumbai, India, while acetonitrile (HPLC grade) and triethylamine (HPLC grade) 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 Retigabine formulation was procured from local market. TROBALT- tablets 500 mg are manufactured by Glaxo Smith Kline Pvt. Ltd.

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 C_{18} 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:
Retigabine was analyzed by various reversed phase columns like C_8 and C_{18} columns. Among C_8 and C_{18} columns, C_{18} (250 mm X 4.6 mm, 5 µm) column was selected. Various combinations of acetonitrile, phosphate buffer and methanol with triethylamine as column modifier were tested. 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 and UV detection wavelength was 302.8 nm with a flow rate of 1 $mL.min^{-1}$. Injection volume was 20 µL, with ambient temperature, run time was 8 minutes and retention time was 5.670 minutes. The resulting HPLC chromatogram was shown in Fig. 3.

Mobile phase preparation:
Accurately 6.056 g of potassium dihydrogen orthophosphate is weighed and dissolved in 445 ml of water to get 10 millimolar phosphate buffer. To this 55 ml of 0.1M ortho phosphoric acid is added and pH was adjusted to 3.0 with triethyl amine. Above prepared buffer and Acetonitrile were mixed in the proportion of 50:50 v/v. The mobile phase so prepared was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

Preparation of standard drug solution:
About 10 mg of Retigabine was correctly weighed and dissolved in 10 ml of mobile phase. This solution was diluted to get concentration of 100µg/ml and then it is further diluted to obtain different concentrations (2, 4, 6, 8, and 10 µg/ml) of Retigabine.

Preparation of Sample solution:
The content of 10 tablets of TROBALT-50 were exactly weighed and transferred into a mortar and ground to a fine powder. From this, tablet powder which is equivalent to 100 mg of Retigabine was taken and the drug was extracted in 100 mL of mobile phase. The resulting solution was filtered using 0.45 µm membrane filter paper and degassed by sonication. This solution was further suitably diluted for chromatography.

Selection of detection wavelength:
For the selection of analytical wavelength 2 µg/mL Retigabine solution was prepared by suitable dilution from standard solution and scanned in the range of 200 to 400 nm. From the spectrum λ_{max} of Retigabine 302.8 was selected for the analysis.

Calibration curve for Retigabine:
Replicates of each calibration standard solutions (2, 4, 6, 8, 10 µg/mL) were injected utilizing a 20µl fixed loop system and the chromatograms were recorded. Calibration curves were constructed by plotting concentration of Retigabine on X-axis and peak areas of standard Retigabine on Y-axis and shown in Figure 2 and regression equations were computed for Retigabine. The calibration data is presented in Table 1.

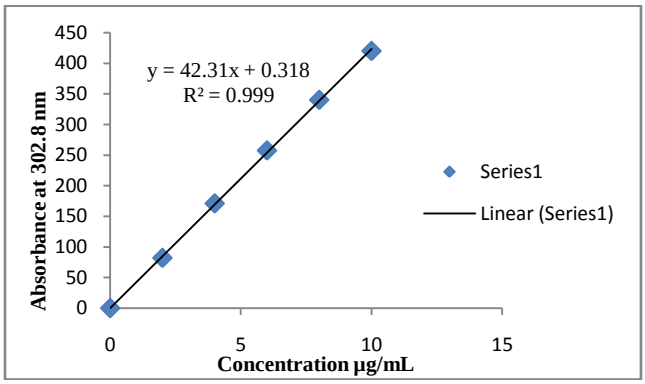

Figure 2: Calibration curve of Retigabine

Table 1: Linear regression data of the proposed HPLC method of Retigabine

Parameter	Method
Detection wavelength(λ max)	By UV at 302.8 nm
Linearity range (µg/ml)	2-10 $\mu g.mL^{-1}$
Regression equation (Y=a+bx)	Y = 42.31x + 0.318
Slope(b)	42.31
Intercept(a)	0.318
Standard deviation of slope (S_b)	0.3512
Standard deviation of intercept (S_a)	2.1268
Standard error of estimation (Se)	2.9386
Correlation coefficient (r^2)	0.9997

% Relative standard deviation* i.e., Coefficient of variation(CV)	0.1305
Percentage range of errors* (Confidence limits) 0.05 significance level 0.01 significance level	 0.4179 0.5493

*Average of 6 determinations; Acceptance criteria < 2.0.

VALIDATION OF THE PROPOSED METHOD

The developed method of analysis was validated as per the ICH Q2 (R1)[15] for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness and system suitability, limit of detection (LOD) and limit of quantitation (LOQ).

System suitability:

System suitability tests are an integral part of chromatographic method which was used to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug solution at the concentration level 10 µg mL^{-1} for Retigabine to check the reproducibility of the system.

At first the HPLC system was stabilized for 40 min. One blank followed by six replicates of a single calibration standard solution of Retigabine was injected to check the system suitability. To ascertain the systems suitability for the proposed method, the parameters such as theoretical plates, peak asymmetry, retention time and parameters were taken and results are presented in Table 2.

Table 2: Optimized chromatographic conditions and system suitability parameters for proposed HPLC method for Retigabine

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C_{18} Column (4.6 X 250 mm, 5 µm)
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 $mL.min^{-1}$.
Detection wave length	By UV at 302.8 nm.
Run time	8 minutes
Column back pressure	98 kgf
Temperature	Ambient temperature (25°C)
Volume of injection loop	20 µL
Retention time (R_t)	5.670 min
Theoretical plates [th.pl] (Efficiency)	17,831
Theoretical plates per meter [t.p/m]	356629
Tailing factor (asymmetry factor)	1.115

Specificity:

The effect of wide range of excipients and other additives usually present in the formulations of Retigabine in the determinations under optimum conditions was investigated. The specificity of the RP-HPLC method was established by injecting the mobile phase and placebo solution in triplicate and recording the chromatograms. The common excepients 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 3.

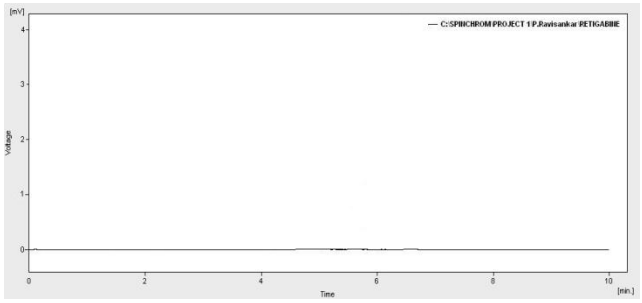

Figure 3: Chromatogram of placebo.

Table 3: Specificity study

Name of the solution	Retention time (R_t) min.
Mobile phase	No peaks
Placebo	No peaks
Retigabine 2 $\mu g.mL^{-1}$	5.670 min.

Linearity:

The linearity graphs for the proposed assay methods were obtained over the concentration range of 2 -10 $\mu g.mL^{-1}$ of Retigabine. Calibration data values and the results are presented in Table 4. The representative chromatograms of 2 -10 $\mu g.mL^{-1}$ indicating the Retigabine are shown in Figure 4 to 8 and the delegate standard and sample chromatograms of Retigabine are shown in Figure 9 and 10 respectively.

Table 4: Calibration data of the proposed HPLC method of Retigabine

S.No	Concentration, $\mu g.mL^{-1}$.	Retention time, (R_t) min.	Peak area, mV.s.
1	0	-	0
2	2	5.670	82.150
3	4	5.670	171.10
4	6	5.670	257.63
5	8	5.670	340.31
6	10	5.670	420.24

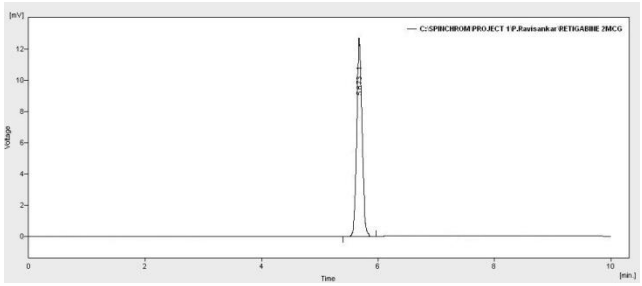

Figure 4: Standard chromatogram of Retigabine (2 $\mu g.mL^{-1}$).

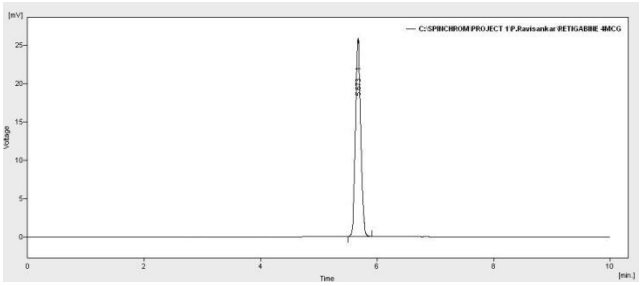

Fig. 5: Standard chromatogram of Retigabine (4 µg.mL^{-1}).

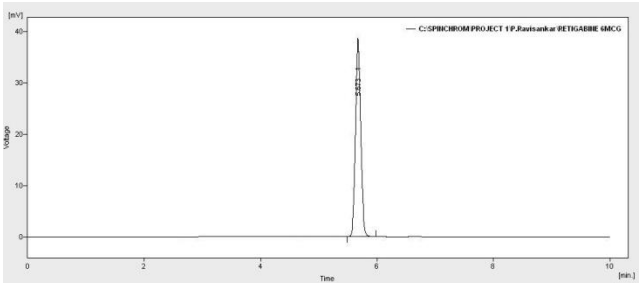

Fig. 5: Standard chromatogram of Retigabine (6 µg.mL^{-1}).

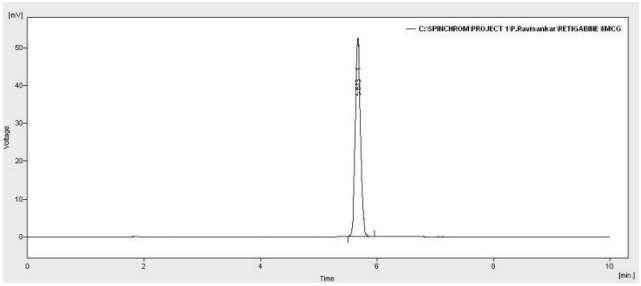

Fig. 5: Standard chromatogram of Retigabine (8 µg.mL^{-1}).

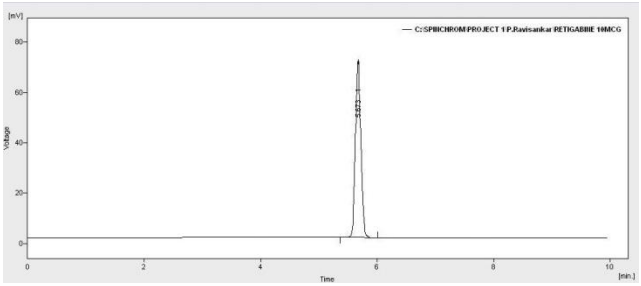

Fig. 5: Standard chromatogram of Retigabine (10 µg.mL^{-1}).

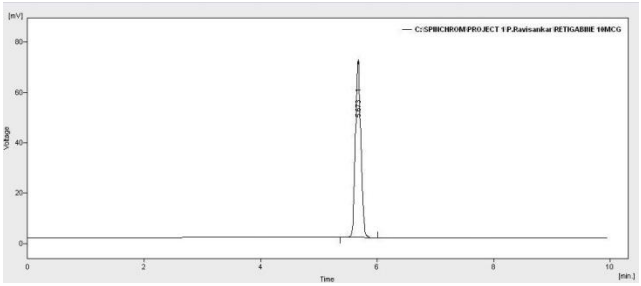

Fig. 9: A typical chromatogram of Retigabine standard

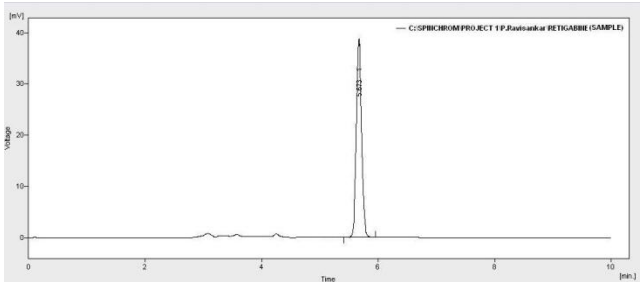

Fig. 10: Chromatogram of market formulation (TROBALT- 50 mg Tablets) of Retigabine.

Precision:

Intra-day and inter-day precision study of Retigabine was carried out by estimating corresponding responses 3 times on the same day and on 3 different days for the concentration of 2 µg/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.

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

Sample	Concentration (µg.mL^{-1})	Injection no.	Peak area	%RSD(acceptance criteria < 2.0)
Retigabine	2	1	82.15	1.02558
		2	81.1	
		3	80.09	
		4	82.14	
		5	81.13	
		6	82.12	

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

Sample	Concentration (µg.mL^{-1})	Injection number	Peak area	%RSD (acceptance criteria < 2.0)
Retigabine	2	1	82.15	1.119039
		2	80.12	
		3	80.11	
		4	81.12	
		5	81.14	
		6	82.14	

Accuracy (Recovery studies):

The accuracy of the method was determined by calculating recovery of Retigabine by the method of addition. Known amount of Retigabine at 80 %, 100 % and 120 % 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 Retigabine at each level was not less than 99 % and not more than 101 %. The results are presented in Table 7.

Table 7: Recovery data of the proposed Retigabine by RP-HPLC method

Recovery level	Amount taken (mg)	amount added (mg)	Total Amount (mg)	% recovery (mg)	Mean % Recovery	%RSD #
80 %	8	5	13	12.95	99.61	0.26
100 %	10	5	15	14.98	99.86	0.17
120 %	12	5	17	16.96	99.76	0.060

#acceptance criteria < 2.0

Robustness:

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

Table 8: Robustness results of Retigabine

S. No	Parameter	Optimized	Used	Retention time (R_t), min	Plate count	Peak asymmetry#	Remark
1.	Flow rate (±0.2 mL. min^{-1})	1.0 mL. min^{-1}	0.8 mL. min^{-1}	5.670	17825	1.121	*Robust
			1.0 mL. min^{-1}	5.665	17818	1.122	*Robust
			1.2 mL. min^{-1}	5.660	17812	1.124	*Robust
2.	Detection wave length (±5 nm)	302.8 nm	300 nm	5.665	17810	1.123	*Robust
			302.8 nm	5.650	17810	1.122	*Robust
			304 nm	5.665	17810	1.122	*Robust
3.	Mobie phase composition (±5 %)	50:50 v/v	55:45 v/v	5.669	17823	1.123	*Robust
			50:50 v/v	5.665	17818	1.122	*Robust
			45:55 v/v	5.663	17810	1.121	*Robust

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 quantization 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 and the assay results of Retigabine formulation are existed in Table 10.

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

Parameter	Results
Limit of Detection (LOD)	0.1658 µg.mL^{-1}
Limit of Quantitation (LOQ)	0.5025 µg.mL^{-1}

Table 10: Assay results of Retigabine formulation

S.No	Formulations	Labelled amount	Amount found	% Assay ±RSD*
1	TROBALT	50 mg	49.962 mg	99.905±1.05

***** Average of 6 determinations.

RESULTS AND DISCUSSION

The mobile phase consisting of 10 mM phosphate buffer (pH-3.0): acetonitrile (50:50 % v/v at 1 mL.min^{-1} flow rate was optimized which gave sharp peak, minimum tailing factor with short run time for Retigabine. The retention time for Retigabine was 5.670 min. UV spectra of Retigabine showed that the drug absorbed maximum at 302.8 nm, so this wavelength was selected as the detection wavelength. The calibration curve for Retigabine was found to be linear over the range of 2-10 µg.mL^{-1}. The data of regression analysis are shown in Table 1. System suitability parameters & optimized chromatographic conditions are shown in Table 2. The developed method was applied to the assay of Retigabine tablets. The experimental results are given in Table 3 and the calibration data are given in Table 4. The results were very close to labelled value of commercial tablets. The regression equation was found to be Y = 42.31x + 0.318 with correlation coefficient is r^2=0.9997 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 Figure 3 with sample peak. The representative chromatograms indicating the Retigabine are shown in Figure. 4 to 8. The representative standard and sample chromatograms of Retigabine are shown in Fig. 9 and 10 respectively. They do not disturb the elution or quantification of Retigabine, 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. Precision was studied to find out intra and inter day variations in the test methods of Retigabine for the three times on the same day and different day. The intra-day and inter-day precision obtained was % RSD (< 2.0) indicates that the proposed method is quite precise and reproducible and results are shown in Tables 5 and 6. Recovery studies of the drug were carried out for the accuracy parameter at three different concentrations levels i.e., multiple level recovery studies. A known amount of Retigabine standard was added into pre-analyzed sample and subjected them to the proposed HPLC method. The % recovery was found to be within the limits as listed in Table 7. Generally the mean percentage recovery of Retigabine at each level was not less than 99 % and not more than 101 %. In this case percentage recovery of Retigabine was found to be in the range of 99.60 % to 99.80 %. The method precision was done and the low %RSD values indicates that the proposed method which was in good agreement with precision. Robustness was done by small changes in the chromatographic conditions like mobile phase flow rate, temperature, mobile phase composition etc., It was observed that there were no marked changes in the chromatograms. 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 limit of detection (LOD) was 0.1658 µg/mL and the limit of quantitation (LOQ) was 0.5025 µg/mL which shows that this method is very sensitive. The results are presented in Table 9 and finally the assay results of Retigabine formulation are existed in Table 10.

CONCLUSION

This paper describes first RP-HPLC method developed and fully validated for the quantitative determination of Retigabine 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 Retigabine in active pharmaceutical ingredient API and pharmaceutical preparations.

ACKNWOLEDGEMENT

The authors thank to Hetero Labs Limited, Jeedimetla, Hyderabad for providing Retigabine as gift sample for this work. They also thank chairman Dr. L. Rathaiah, Vignan Pharmacy College and P. Srinivasa Babu, Principal of Vignan Pharmacy College for providing necessary facilities to carry out this research work.

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