

AN ANALYTICAL NEW RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ANTI-VIRAL DRUGS DARUNAVIR AND COBICISTAT IN BULK FORM AND MARKETED PHARMACEUTICAL TABLET DOSAGE FORM

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ABSTRACT: A new, simple, rapid, precise, accurate and reproducible RP-HPLC method for estimation of Brivaracetam in bulk form and marketed formulation. Separation of Brivaracetam was successfully achieved on a Symmetry ODS (C18) RP Column, 250 mm x 4.6 mm, 5 μ m column in an isocratic mode of separation utilizing Water: Methanol in the ratio of 20:80% v/v at a flow rate of 1.0 mL/min and the detection was carried out at 284nm. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The response was found to be linear in the drug concentration range of 60-140mcg/mL for Brivaracetam. The correlation coefficient was found to be 0.9995 for Brivaracetam. The LOD and LOQ for Brivaracetam were found to be 0.07 μ g/mL and 0.21 μ g/mL respectively. The proposed method was found to be good percentage recovery for Brivaracetam, which indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard solution with the sample solution. Therefore, the proposed method specifically determines the analyte in the sample without interference from excipients of pharmaceutical dosage forms.
Keywords: Brivaracetam, RP-HPLC, Accuracy, ICH Guidelines, Precision.

I. INTRODUCTION

Brivaracetam is a racetam derivative of Levetiracetam used in the treatment of partial-onset seizures. Brivaracetam binds SV2A with 20 times higher affinity than Levetiracetam. It is available under the brand name Briviact made by UCB. Brivaracetam¹ is an orally bioavailable Levetiracetam derivative, with anticonvulsant activity. Although the exact mechanism through which Brivaracetam exerts its effects is not fully known, this agent targets and binds to synaptic vesicle protein 2A (SV2A) in the brain. This prevents synaptic vesicle exocytosis and the synaptic release of certain, as of yet not fully known, excitatory neurotransmitters. This may inhibit impulse conduction across synapses, decrease neuronal (hyper-) excitability, and may modulate epileptogenesis. SV2A, a membrane glycoprotein present in neuronal synaptic vesicles, plays a key role in action potential-induced neurotransmitter release in the brain. Brivaracetam² binds SV2A with high affinity. SV2A is known to play a role in epileptogenesis through modulation of synaptic GABA release. It is thought that Brivaracetam exerts its anti-epileptogenic effects through its binding to SV2A. Brivaracetam is also known to inhibit Na⁺ channels which may also contribute to its anti-epileptogenic action. The precise mechanism of Brivaracetam's anti-epileptogenic activity is unknown. Brivaracetam³ is believed to act by binding to the ubiquitous synaptic vesicle glycoprotein 2A (SV2A), like Levetiracetam but with 20-fold greater affinity. There is some evidence that racetams including Levetiracetam and Brivaracetam access the luminal side of recycling synaptic vesicles during vesicular endocytosis. They may reduce excitatory neurotransmitter release and enhance synaptic depression during trains of high-frequency activity, such as is believed to occur during epileptic activity. The IUPAC Name of Brivaracetam is (2S)-2-[(4R)-2-oxo-4-propyl pyrrolidin-1-yl] butanamide. The Chemical Structure of Brivaracetam is follows

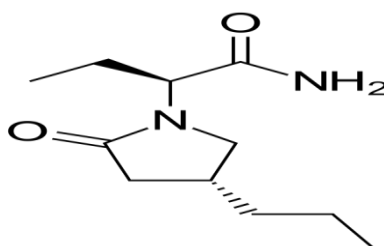


Fig.1. Chemical Structure of Brivaracetam

II. EXPERIMENTAL

Table-1: List of Instrument used

S. No.	Instruments/Equipments/Apparatus
1.	HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	ELICO SL-159 UV – Vis spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry ODS RP C ₁₈ , 5 μ m, 15mm x 4.6mm i.d.
7.	P ^H Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

Table-2: List of Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai
7.	Ethanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai
8.	Octanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Selection of Wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase⁴ diluting with the same solvent.(After optimization of all conditions) for UV analysis. It scanned in the UV spectrum⁵ in the range of 200 to 400nm. This has been performed to know the maxima of Brivaracetam, so that the same wave number can be utilized in HPLC UV detector⁶ for estimating the Brivaracetam. The scanned UV spectrum is attached in the following page,

Table-3: Summary of Process Optimization

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Symmetry ODS (C ₁₈) RP Column, 250 mm x 4.6 mm, 5 μ m	Methanol : Acetonitrile = 20 : 80	1.0ml/min	284nm	Very Low response	Method rejected

Symmetry ODS (C ₁₈) RP Column, 250 mm x 4.6 mm, 5µm	Methanol : Water = 50 : 50	1.0ml/min	284nm	Low response	Method rejected
Symmetry ODS (C ₁₈) RP Column, 250 mm x 4.6 mm, 5µm	Acetonitrile: Water = 80 : 20	1.0ml/min	284nm	Tailing peaks	Method rejected
Symmetry ODS (C ₁₈) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer : Acetonitrile = 75:25 (pH-4.8)	1.0ml/min	284nm	Resolution was not good	Method rejected
Symmetry ODS (C ₁₈) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer : Methanol = 40:60 (pH-4.0)	1.0ml/min	284nm	Tailing peak	Method rejected
Symmetry ODS (C ₁₈) RP Column, 250 mm x 4.6 mm, 5µm	Water : Methanol = 20:80	1.0ml/min	284nm	Nice peak	Method accepted

Preparation of Mobile Phase:

200ml of HPLC Grade Water and 800ml of HPLC Grade Methanol were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration⁷.

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Brivaracetam working standard into a 10ml of clean dry volumetric flasks add about 7ml of diluent. Then sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 1ml of the above Brivaracetam stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution:

Take average weight of Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Brivaracetam sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 1ml of the above Brivaracetam stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Method Validation

Analytical method validation⁸⁻¹⁰ establishes documented evidence that the procedure adopted for a test is fit for the intended purpose in terms of quality, reliability and consistency of results.

Selectivity and Specificity

Selectivity¹¹ and specificity¹² are often used synonymously but these are different terms. Specificity is the unequivocal response of the technique to a specific analyte present in the sample whereas selectivity can be applicable to a collective response to a group of analytes having similar chemical and physical characteristics.

Linearity

Linearity¹³ refers to the ability of analytical procedures to produce results in direct proportion to the concentration range of analyte in samples within the required concentration levels.

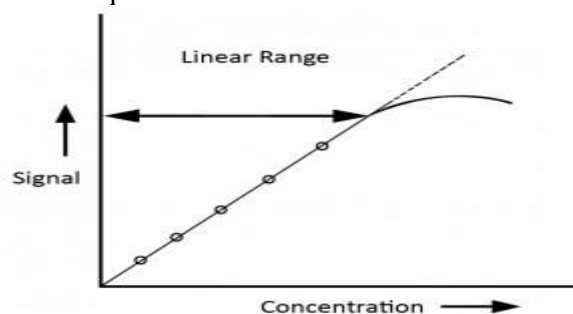


Fig.2. Linearity Graph

Linearity should be determined using a minimum of 6 standards whose concentration spans from 80% to 120% of expected concentration level¹⁴.

Linearity report should include slope of line, linear range and correlation coefficient data. Correlation coefficient¹⁵

r should be greater than or equal to 0.99 in the working range.

Range

Operating range is deduced from the calibration plot. It is the interval between the upper and lower concentration of analyte falling in the linear range¹⁶. The results corresponding to this range demonstrate acceptable levels of precision, accuracy and linearity.

Accuracy

Degree to which the determine value of analyte corresponds to the true value.

Accuracy¹⁷ can vary over the expected concentration range.

It should be determined using a working or reference standards in the 80% – 120% level of expected range¹⁸.

Accuracy is determined by:

- ✓ Analyzing a sample of known concentration and comparing with the true value
- ✓ Spiking a blank (Sample having all components except the analyte) and comparing with the expected result.
- ✓ Standard addition method in which the sample concentration is determined. A known amount of analyte is added and the concentration is once again determined. The difference of the two concentration values is compared with the actual value¹⁹ of added analyte.

Precision

Precision expresses closeness of a series of measurements of the same sample under identical conditions

High degree of precision²⁰ does not necessarily means a high degree of accuracy

Precision is expressed as variance, standard deviation or as coefficient of variation²¹ of a series of measurements

Minimum of five replicate sample determinations should be carried out

Limit of Detection

Lowest amount of an analyte that can be detected but not necessarily quantified

Lowest concentration of calibration standard²² which produces a peak response corresponding to the analyte should be measured at least 6 to 10 times. Average response (X) and standard deviation²³ (SD) are required to calculate limit of detection

$$\text{Limit of detection} = X + (3SD)$$

Signal to noise ratio at limit of detection should be at least 3:1

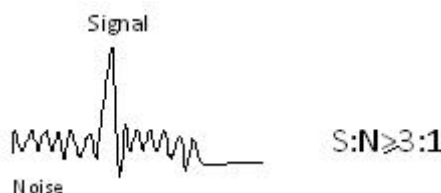


Fig.3. Signal to noise ratio should be greater than 3 at limit of detection and greater than 10 at limit of quantification

Limit of Quantitation

Lowest amount²⁴ of the analyte that can be quantitatively determined with defined precision under the stated experimental conditions

6 – 10 observations should be made for average response and standard deviation

$$\text{Limit of quantitation} = X + (10SD)$$

Signal to noise ratio should be at least 10:1 at the limit of quantitation

Ruggedness

Ruggedness²⁵ measures reproducibility of test results under following conditions:

- ✓ Results generated for same sample under identical conditions by different laboratories
- ✓ Results generated for sample under identical conditions by different analysts²⁶
- ✓ Same analysis using different instruments
- ✓ Same analysis under different environmental conditions
- ✓ Same analysis using test materials from different sources

Robustness

Robustness²⁷ examines the effect of operational parameters changes on the analytical results

- pH
- Temperature
- Operational conditions such as flow rate, injection volume, detection wavelength²⁸ or mobile phase

composition in chromatographic analysis

Variation should be deliberate but within realistic range²⁹ to study the robustness of the method. The results of the analysis after making the deliberate changes should be within the method’s specified tolerance limits.

III. RESULTS AND DISCUSSION

Selection of Wavelength

The scanned UV spectrum is attached in the following page,

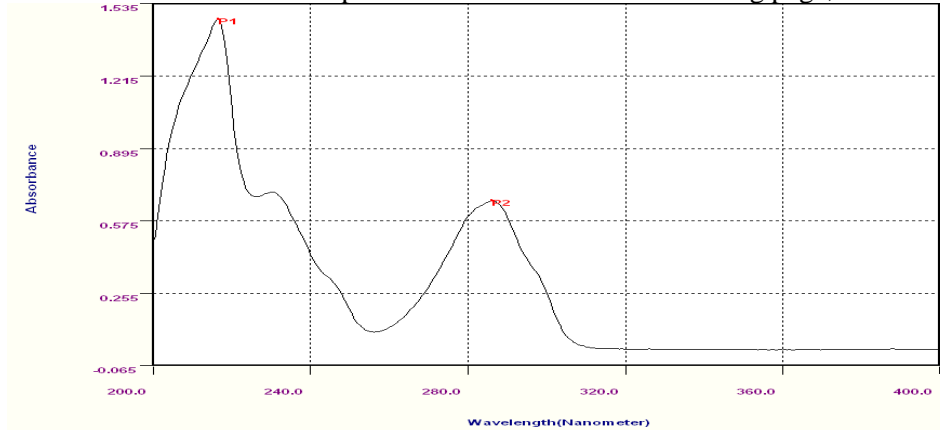


Fig.4. UV Spectrum for Brivaracetam

Summary of Optimized Chromatographic Conditions

The Optimum Chromatographic conditions³⁰ obtained from experiments can be summarized as below:

Table-4: Summary of optimized Chromatographic conditions

Mobile phase	Water : Methanol (20:80v/v)
Column	Symmetry ODS (C ₁₈) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	284 nm
Flow rate	1.0 ml/ min.
Run time	10 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic
Retention time	4.783 minutes

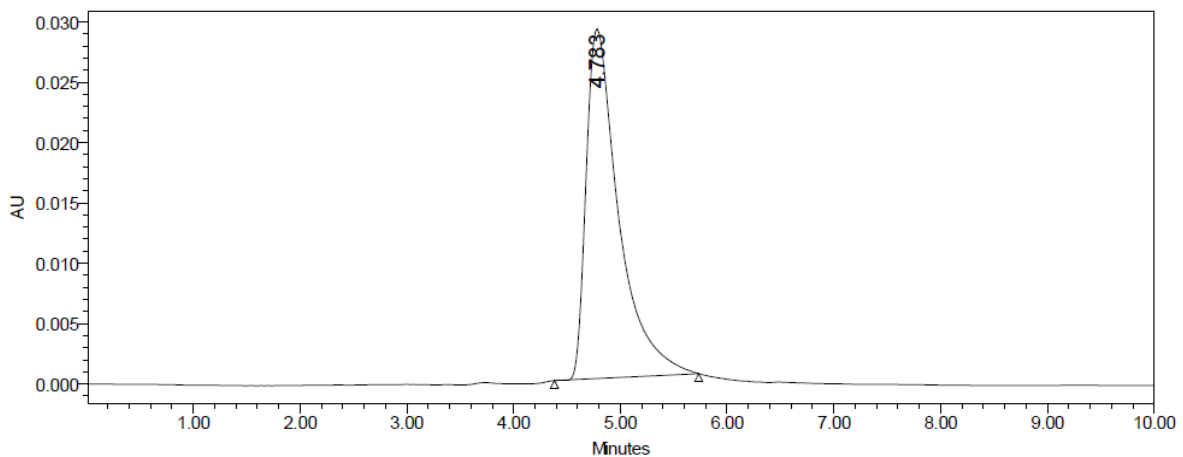


Fig.5. Chromatogram of Brivaracetam in Optimized Condition

Observation: The selected and optimized mobile phase was Water: Methanol = 20:80 and conditions optimized

were flow rate (1.0 ml/minute), wavelength (284nm), Run time was 10 mins. Here the peaks were separated and showed better resolution, theoretical plate count and symmetry. The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

Validation of Analytical Method

1. Linearity:

To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 60-140 μ g/ml. The prepared solutions were sonicated. From these solutions, 10 μ l injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

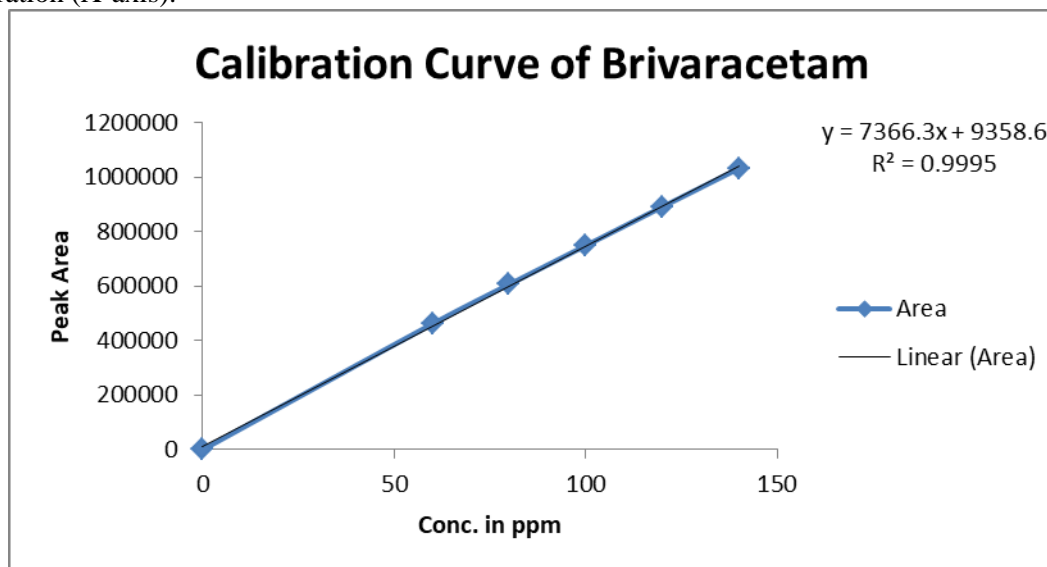


Fig.6. Calibration Curve of Brivaracetam

Table-5: Linearity Data for Brivaracetam

Conc. (μ g/ml)	Area
0	0
60	461404
80	606157
100	748506
120	891041
140	1032196

2. Accuracy: The accuracy of the method was determined by recovery studies and the percentage recovery was calculated. The recoveries of Brivaracetam were found to be in the range of 99.91 %. The proposed Liquid Chromatographic method was applied to the determination of Brivaracetam. The results for Brivaracetam comparable with the corresponding labeled amounts.

Table-6: Shown Accuracy Observation of Brivaracetam

Accuracy	Amount Added	Amount Recovered	Peak Area	% Recovery	Mean Recovery
80%	80	80.798	604517	100.997	99.6%
	80	80.673	603598	100.841	
	80	80.756	604213	100.945	
100%	100	99.933	745471	99.933	
	100	100.083	746574	100.083	
	100	100.365	748652	100.365	
120%	120	120.290	895415	100.241	
	120	120.201	894762	100.167	
	120	120.442	896541	100.368	

Precision:**Repeatability:**

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Brivaracetam (API). The percent relative standard deviation was calculated for Brivaracetam are presented in the table-7.

Table-7: Repeatability Data for Brivaracetam

S. No.	INJECTION	PEAK AREA
1	Injection 1	743826
2	Injection 2	745277
3	Injection 3	742506
4	Injection 4	747576
5	Injection 5	746715
6	Injection 6	741278
7	Average	744529.6667
8	SD	2440.4116
9	% RSD	0.32777

Intermediate Precision:

The Intermediate Precision consists of two methods:-

Intra Day: In Intra Day process, the 80%, 100% and 120% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 80%, 100% and 120% concentration are injected at same intervals of time in different days.

Table-8: Results of intra-assay & inter-assay

Conc. of Brivaracetam (API) (µg/ml)	Observed Conc. of Brivaracetam (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
80	80.38	0.56	80.45	0.56
100	100.17	0.71	100.50	0.77
120	120.89	0.89	120.91	0.85

Observations: The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Brivaracetam revealed that the proposed method is precise.

4. LOD and LOQ:

The LOD and LOQ parameter was evaluated by mistreatment the slope of line and variance obtained from accuracy studies.

The detection limit (LOD) and quantization limit (LOQ) may be expressed as:

$$\text{L.O.D.} = 3.3(\text{SD}/\text{S}).$$

$$\text{L.O.Q.} = 10(\text{SD}/\text{S})$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.07 & 0.21 µg/ml respectively.

5. System Suitability:

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that

the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-9.

Table-9: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Retention Time	RT > 2	Brivaracetam=4.783
2	Asymmetry	T ≤ 2	Brivaracetam=1.35
3	Theoretical plate	N > 2000	Brivaracetam=2865
4	Tailing Factor	T < 2	Brivaracetam=1.37

6. Method Robustness: Influence of small changes in chromatographic conditions such as change in flow rate 1.0 ml (± 0.1 ml/min), Wavelength of detection 284 (± 2 nm) & organic phase content in mobile phase ($\pm 5\%$) studied to determine the robustness of the method are also in favour of (Table-10, % RSD < 2%) the developed RP-HPLC method for the analysis of Brivaracetam (API).

Table-10: Result of method Robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.45
Flow (0.9 ml/min)	0.38
More Organic	0.76
Less Organic	0.65
Wavelength of Detection (286 nm)	0.98
Wavelength of detection (282 nm)	0.93

7. Estimation of Brivaracetam in Pharmaceutical Dosage Form

Twenty Tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 25 mg of drugs were transferred to 25 ml volumetric flask, make and solution was sonicated for 15 minutes, there after volume was made up to 25 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with mobile phase. The solution was filtered through a membrane filter (0.45 μ m) and sonicated to degas. The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.

The data are shown in Table-11.

ASSAY:

Assay % =

$$\frac{\text{AT}}{\text{AS}} \times \frac{\text{WS}}{\text{DS}} \times \frac{\text{DT}}{\text{WT}} \times \frac{\text{P}}{100} \times \text{Avg. Wt} = \text{mg}$$

Where:

AT = Peak Area of drug obtained with test preparation

AS = Peak Area of drug obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

Table-11: Recovery Data for estimation Brivaracetam in Briviact

Brand name of Brivaracetam	Labelled amount of Drug (mg)	Mean (\pm SD) amount (mg) found by the proposed method (n=6)	Assay % (\pm SD)
Briviact 50mg Tablet (UCB India Pvt Ltd)	50mg	49.867 (\pm 0.468)	99.825 (\pm 0.418)

Result & Discussion: The amount of drug in Briviact Tablet was found to be 49.867 (\pm 0.468) mg/tab for Brivaracetam & %Purity was 99.825 %.

Stability Studies

The results of the strain studies indicated the specificity of the tactic that has been developed. Brivaracetam was stable in oxidation and thermal stress conditions. The result of forced degradation studies are given in the following table-12.

Table-12: Results of forced degradation studies of Brivaracetam

Stress Condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	90.14	9.86	100.0
Basic Hydrolysis (0.1 M NaOH)	24Hrs.	96.46	3.54	100.0
Wet heat	24Hrs.	98.33	1.67	100.0
UV (254nm)	24Hrs.	92.02	7.98	100.0
3 % Hydrogen peroxide	24Hrs.	91.36	8.64	100.0

IV. SUMMARY AND DISCUSSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Brivaracetam, different chromatographic conditions were applied & the results observed are presented in previous chapters.

Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution.

In case of RP-HPLC various columns are available, but here Symmetry ODS (C18) RP Column, 250 mm x 4.6 mm, 5 μ m column was preferred because using this column peak shape, resolution and absorbance were good.

Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Brivaracetam it is evident that most of the HPLC work can be accomplished in the wavelength range of 284 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 10 μ l were found to be the best analysis.

The result shows the developed method is yet another suitable method for assay which can help in the analysis of Brivaracetam in different formulations.

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