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# DEVELOPMENT OF BIOANALYTICAL METHOD AND VALIDATION FOR QUANTIFICATION OF BRIVARACETAM IN HUMAN PLASMA BY RP-HPLC : RESEARCH ARTICLE

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## Abstract:

High-Performance Liquid Chromatography is an analytical method that has been developed and validated for the estimation of Brivaracetam in Human Plasma according to current regulatory guidelines for bioanalytical methods. Sample processing involved a simple protein precipitation extraction technique by acetonitrile as the extraction solvent. Methods: Separation is carried out on Sunfire-C18 plasma 5 µm (4.6 × 250 mm) column as a stationary phase, Mobile Phase: methanol + 0.1% OPA (orthophosphoric acid) in ratio (45: 55), at a flow rate of 0.7 ml/min. The sample was detected at a wavelength of 279 nm and the sample size was 20µl. The objective of method validation, according to ICH principles, is to show that an analytical technique is appropriate for the intended use. The standard calibration plot was found linear over a range of 10 to 50 µg/ml and the correlation coefficient was found to be ($R^2$ =0.999). The % RSD value of intraday and interday precision is 0.17 and 0.04 respectively. The LOD and LOQ were found to be 0.115034 and 0.34587 for Brivaracetam. The developed method was eventually applied for quantification of the marketed formulation satisfactory result was obtained. Conclusions: It can be concluded that the developed bioanalytical method is capable of quantifying Brivaracetam from spiked human plasma samples. The method meets the requirements of the USFDA Guidelines and can be applied to Bioavailability/ Bioequivalence studies of Brivaracetam.

**Keywords:** Bioanalytical method development, Protein precipitation extraction, Method Validation, Human Plasma, RP-HPLC

# 1.Introduction:

Epilepsy affects more than 50 million people worldwide.[1] There are more than 20 antiepileptic medications (AEDs) that can be used to treat epilepsy and are also approved to treat a variety of seizures or syndromes kinds. However, around a third of individuals do not benefit from AED therapy.[2,3]

Brivaracetam is a white to off-white amorphous powder with a melting point of 72-78$^0$C, and soluble in DMSO (Slightly), and Methanol (Slightly).[4] It is a brand-new antiepileptic drug that has obtained clinical approval to treat individuals with partial-onset seizures in adults and adolescents. The Drug Enforcement Administration places it in the Schedule V classification under the Controlled Substance Act because it has some misuse potential. It is essential to develop a faster, simple, and highly sensitive method for the quantification of Brivaracetam in human plasma by employing simple. BRV, a specific, greater-affinity ligand binding for synaptic vesicle 2A (SV2A), is a membrane protein and galactose carrier.[5] Brivaracetam (BRV) is one of the first AEDs under investigation that was identified during a large-scale clinical research program aimed at optimizing pharmacodynamic efficacy at a new molecular AED target. [6,7] United state food and drug administration (USFDA) approved BRV in 2016 for adjunctive treatment for focal seizures in patients aged ≥16 years with epilepsy (FDA 2016). [8]

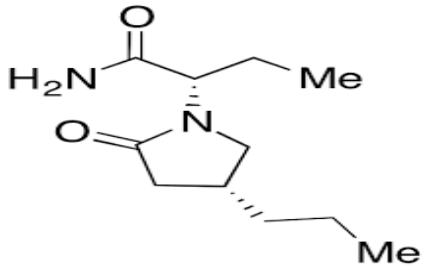
A literature survey revealed that Brivaracetam is estimated by High-performance Liquid Chromatography, liquid chromatographic– ultraviolet estimation, RP-HPLC-PDA, UV Spectrophotometric**,** GC/MS, Liquid chromatography–tandem mass spectrometry with electrospray ionization, LC-MS/MS. Several methods have been reported for the quantification of Brivaracetam in plasma as mentioned above.[9]

The present report of the current investigation for the analysis of Brivaracetam in plasma can be determined using a highly accurate, sensitive, and speedy RP-HPLC technique. The current study's plan is as follows: Optimization of chromatographic conditions were proposed to be developed and optimized like the selection of initial separation conditions, nature of the stationary phase, nature of the mobile phase (pH, peak modifier, solvent strength, ratio, and flowrate). According to US-FDA requirements, it was also suggested that the created method be verified utilizing a variety of validation parameters, including accuracy, precision, selectivity, recovery, and stability.

The HPLC is the most important and essential analytical instrument used in all phases of drug discovery, development, and production. HPLC is the method of choice for determining the peak purity of new chemical entities, monitoring reaction changes during synthetic procedures or scale, assessing new formulations, and performing quality control/assurance of the finished therapeutic product. The major objective of the HPLC approach is to extract and measure the active substance as well as any contaminants from the process, accessible synthetic intermediates, and degradation. [10]

**Drug profile**

**Table no. 1 Drug profile[11]**

| **Sr. NO** | **Name of Drug** | **Brivaracetam** |
|---|---|---|
| **1.** | Structure |  |
| **2.** | Molecular Formula | $C_{11}H_{20}N_2O_2$ |
| **3.** | Molecular Weight | 212.29 g/mol |

| | | |
|---|---|---|
| **4.** | Category | Antiepileptic drugs |
| **5.** | Chemical Name | (2S)-2-[(4R)-2-oxo-4-propyltetrahydro-1H-pyrrol-1-yl] butanamide. |
| **6.** | Description | a white to off-white amorphous powder. |
| **7.** | Melting Point | 72-78 °C |
| **8.** | Solubility | SDMSO (Slightly), Methanol (Slightly) |
| **9.** | Mode of Action | Brivaracetam binds SV2A with high affinity. SV2A is known to play a role in epileptogenesis through modulation of synaptic GABA |

## 2. Material and method

### 2.1. Chemicals and Reagents:

The reference analytical standards for Brivaracetam were procured from Relible's shree laboratory and Research Center (Jalgoan, India). ). Acetonitrile and Methanol (HPLC grade) were procured from Spectrochem Private Ltd. Mumbai (INDIA). Milli-Q (water purification system) (In-house) was used to prepare HPLC-grade water for analysis. Human plasma was collected from Rajashri Chhatrapati Shahu Maharaj blood bank (Kolhapur, India). The tablets [50mg Label Claim] were collected from the medical and the manufacturer was Torrent Pharma and the brand name of the Tablet was Briviact 50 mg (Torrent Pharma Ltd.)

### 2.2. Instrument used, optimized condition

Agilent(1100) Tech. Gradient System with Auto Injector HPLC used for our research project. The optimized chromatographic parameters/conditions were listed in Table 2. Following chromatographic condition were established by trial & error method and were kept constant throughout the examination

**Table 2. HPLC conditions.**

| **HPLC Conditions** | |
|---|---|
| Mobile Phase | Methanol + 0.1% OPA (45:55%v/v) |
| Column | 4.6 × 250 mm |
| Stationary phase | Sunfire C18 |
| Dectector | UV (DAD) G13148 S.No. DE711365875 |
| Autosampler Temperature | 25 $^{0}$C |
| Detection wavelength | 279 nm |
| Flow rate | 0.7 ml/min |
| Injection volume | 20 µl |

### 2.3. Stock and Working solution Preparation

HPLC Grade Methanol was Utilized for preparing the Standard stock solution of Brivaracetam and Weigh accurately 10 mg of Brivaracetam API dilute with 10 ml Methanol i.e 1000 µgm/ml this API stock is used for trials in method development. Above Stock solution measure 0.3 ml and dilute with 10 ml of methanol i.e. 10 µgm/ml

**Preparation of 0.1% OPA:**

0.1% OPA was prepared by taking 1 ml of OPA in 1000 ml HPLC grade water. Mix buffer, acetonitrile, and methanol in the ratio of 40:40:20.

**Preparation of mobile phase:**

The mobile phase was prepared by mixing the above Methanol [HPLC grade] 450mL (45%) and 550 mL of 0.1% OPA (55%) and degassed in an ultrasonic water bath for 5 minutes. Then the solution was filtered through 0.45µm filter under vacuum filtration

**Selection of Wavelength:**

Using UV spectrophotometry, the spectrum was captured by scanning dilutions of reference solutions in the 200 nm region. The approach demonstrated good linearity in this range with a detection wavelength of 279 nm.

**Selection of Flow rate:**

The flow rate of 0.7 ml/min was chosen to have a high flow rate and reduce retention time, allowing component molecules to travel through the column fast with limited opportunity to interact with the stationary phase.

**Selection of Stationary phase:**

Sunfire C18 (Agilent)2 stationary phases are used for separation because of its hydrophobic interaction. Hydrocarbons in the stationary phase can attract and hold solutes in the mobile phase as they pass through the pores in the silica (Agilent). The mobile phase consisted of methanol and 0.1% OPA in a 45:55 ratio, and the detection wavelength was chosen to be 279 nm. The flow rate for separation was 0.7 ml/min. The column's temperature was 25°C, and the particles were 20 µl in size.

**Diluent Preparation:**

The Mobile phase was used as diluent.

Storage of the stock solutions and stock dilutions was done at room temperature during their usage

**Preparation of Standard Brivaracetam Solution:** The Standard Stock Solution was prepared by weighing accurately and transferring 10mg of Brivaracetam [Working standard] in 10 ml Methanol into a 10 mL volumetric flask. Initially about 10 mL of diluent was added and sonicated to dissolve it completely and the volume was make up to the mark with the same solvent. Further from the above Stock Solution pipette out 0.3 ml solution into a 10 ml volumetric flask and diluted up to the mark with diluent. The resultant solution was mixed thoroughly and filtered through a 0.45µm filter.

### 2.4. Sample Processing/ Preparation

Plasma sample were taken out from deep freezer and thawed in a water bath kept at room temperature. It was mixed to completely homogenise the plasma content. The serial dilutions of analyte were prepared in the mobile phase and 0.5ml of each dilution was spiked into 0.5ml of plasma in a polypropylene tube. Then all the tubes were cyclo mixed for 5 min. Then 1 ml of acetonitrile (extraction additive) was added and centrifuged for Approximately 20 min at 3000 rpm. Further the supernatant liquids were collected in another Eppendorf tube. Added 0.5 mL mobile phase to the dry evaporated tubes, vortex mixed with reconstituting and injected onto the HPLC instrument.[12]

### 2.5. Parameters of Method Validation

Method Validation was performed for the developed method following the recent USFDA - Bioanalytical Method Validation guidelines. [11] Validation is a process that involves confirmation or establishment by laboratory studies that a method/procedure/system/analyst can give the required accuracy, precision, sensitivity, ruggedness, etc. In the most basic form, validation of an analytical procedure demonstrates that the procedure developed is suitable for its intended purpose. Validation of the method was carried out after the development of the HPLC method. This section describes the procedure that allowed for the validation of the methods developed.[13]

**Accuracy:**

The accuracy of the drug was calculated by comparing the concentration obtained from the relative recovery of drug supplemented plasma to the actually added concentration. The accuracy was determined by standard

addition method. Three different levels (80%, 100% and 120%) of standards were spiked to commercial tablet in triplicate. The mean of percentage recoveries and the %RSD was calculated

**Linearity:**

The different concentrations of standard solutions were prepared to contain 10-50 µg/mL of Brivaracetam containing 1000 µg/ml of plasma. For quantitative analysis of Brivaracetam, the calibration curves were plotted for each concentration ranges. The linearity ranges for Brivaracetam found to be 10-50 µg/mL respectively Admission Criteria: The calibration's correlation coefficient must be at least 0.99

**Precision:**

The precision of the method was determined by intraday precision and interday precision. The intraday precision was evaluated by analysis of plasma samples containing Brivaracetam at three different concentrations. The interday precision was similarly evaluated over two days period. The reproducibility of proposed method was determined by performing tablet assay at different time intervals (3 hour interval) on same day (Intra-day precision) and on three consecutive days (Inter-day precision). The mean concentration, standard deviation and % CV were calculated.

**System-suitability:**

System suitability was done to verify the repeatability of HPLC method. Theoretical plate, repeatability of retention time and peak area were determined and compared

**Robustness:**

In accordance with the ICH criteria, the robustness of an analytical procedure is measured by its capacity to remain unaffected by slight, intended modifications to the technique parameters and to exhibit its dependability under typical circumstances. A robustness research approach systematically modifies the criteria in order to determine if their results are changed as in:

- Variation in composition of the mobile phase
- Variation in wavelength
- Variation in flow rate

**Limit of detection (LOD) & Limit of Quantification (LOQ):**

The LOD and LOQ of Brivaracetam by the proposed methods were determined using calibration standards. LOD and LOQ values were calculated as 3.3 × SD/D and 10 × SD/D respectively, where D is the slope of the calibration curve and SD is the standard deviation

**Result and Discussion:**

**Results of the investigation of Brivaracetam in plasma by RP-HPLC estimation method**

The present study was carried out to develop a sensitive, precise, and accurate RP-HPLC method for the analysis of the drug Brivaracetam in Human Plasma. In order to method development under Gradient conditions, mixtures of Sodium Dihydrogen Phosphate Buffer with pH 4.0 adjusted with Orthophosphoric acid (OPA) 0.1% and Methanol HPLC grade in different combinations were tested as mobile phase on a Symmetry C18 (4.6 x 250 mm, 5 µm, Make: Thermo) column. A binary mixture of Sodium Dihydrogen Phosphate Buffer [pH 4.0] and Methanol [HPLC Grade] in a 45:55 v/v proportion was proved to be the most suitable of all combinations since the chromatographic peaks were better defined and resolved and almost free from the tailing. The retention times obtained for Brivaracetam were around 4.781 min. A model chromatogram was shown in Fig. no.1 & 3

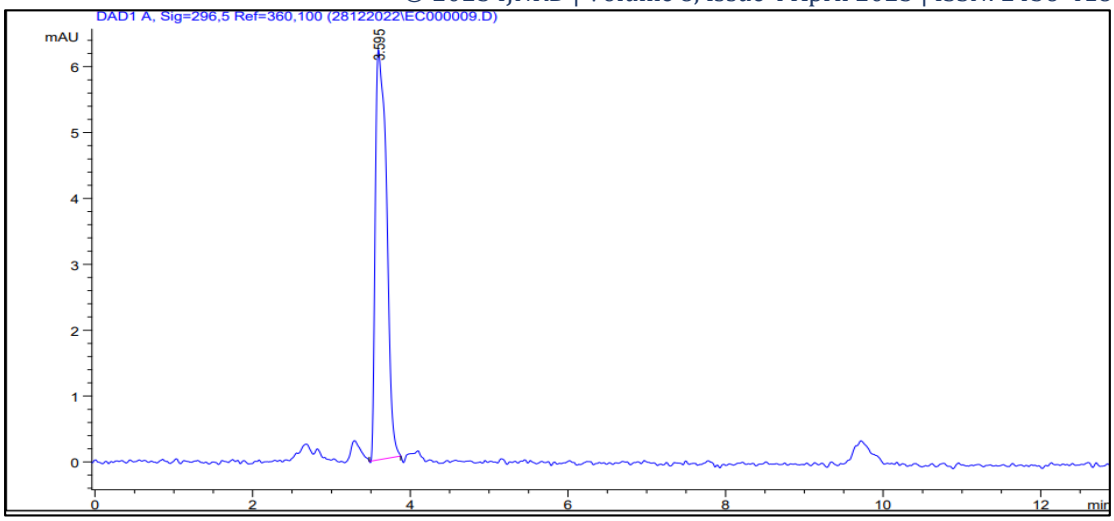



**Fig. No. 1:** Typical chromatogram of blank plasma.

## UV spectroscopy:

Selection of wavelength and calibration curve UV absorption of 10 mg of Brivaracetam was transferred in 100 ml with a sufficient quantity of MeOH generated and absorbance was taken in the range of 200-400 nm. λ max of Brivaracetam in Me was found to be 279 nm.

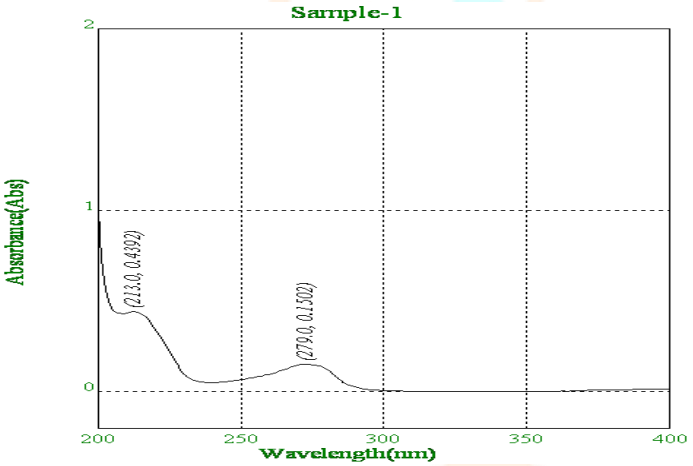



**Fig No.2** UV Visible spectra of Brivaracetam in Methanol

## OPTIMIZED CHROMATOGRAPHIC BATCH:

**Table No. 3 : Optimized Chromatographic Conditions**

| Sr. No | Parameter | Description |
|---|---|---|
| 1 | HPLC | Agilent Tech. Gradient System with Autoinjector |
| 2 | Detector | UV (DAD) G13148 S.NO. DE71365875 |
| 3 | Pump | Quaternary Gradient |
| 4 | Column | 4.6 x 250 mm |
| 5 | Stationary Phase | Sunfire C18 |
| 6 | Mobile Phase | MEOH + 0.1 % OPA ( 45:55 % v/v ) |
| 7 | Detection Wavelength | 279 nm. |
| 8 | Sample size | 20 µl |
| 9 | Flow rate | 0.7 ml/min |

**Fig No.3** Optimized Chromatogram of Brivaracetam in Plasma

**Table No. 4. Optimized method for RP-HPLC**

| Retention time (min) | Area | Height | Symmetry | Width | Plates | Resolution | selectivity |
|---|---|---|---|---|---|---|---|
| 4.781 | 360.50732 | 32.2884 | 0.72 | 0.1667 | 4558 | 3.89 | 1.31 |

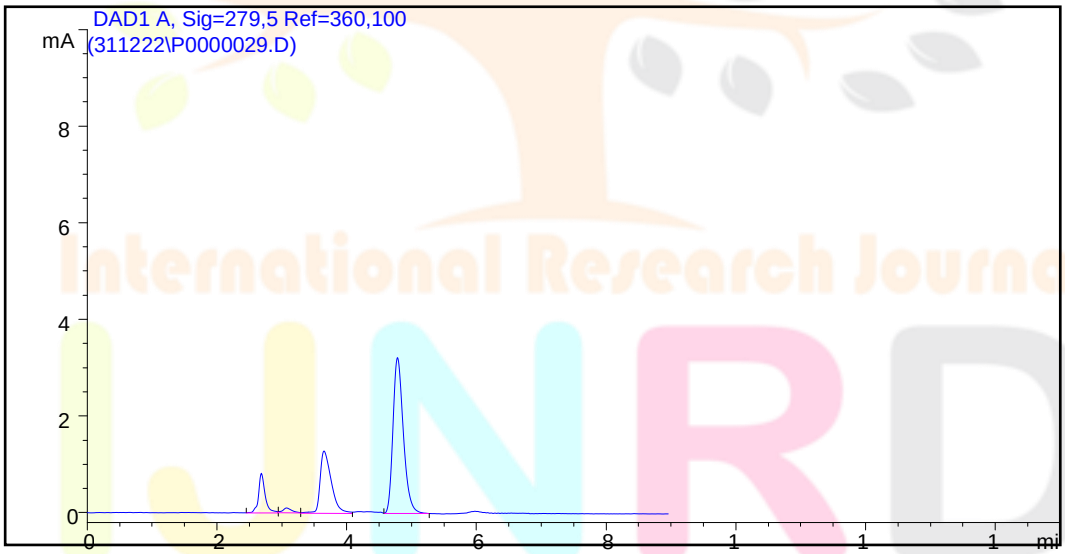
## 1. Method Validation Results:

The developed method was evaluated using ICH guidelines for specificity, linearity, range, accuracy, precision, LOD, LOQ, and robustness.

### A. Linearity

As the concentration of the drug increases area under the curve also increases.

**Table 5 : Linearity results**




| Concentration (ug/ml) | Area | Mean | SD | %RSD |
|---|---|---|---|---|
| 10 | 410.54 | 458.88 | 5.67 | 1.24 |
| 20 | 809.61 | 824.95 | 0.14 | 0.02 |
| 30 | 1245.43 | 1260.77 | 0.16 | 0.01 |
| 40 | 1643.38 | 1658.72 | 0.43 | 0.03 |
| 50 | 2045.20 | 2060.54 | 0.75 | 0.04 |

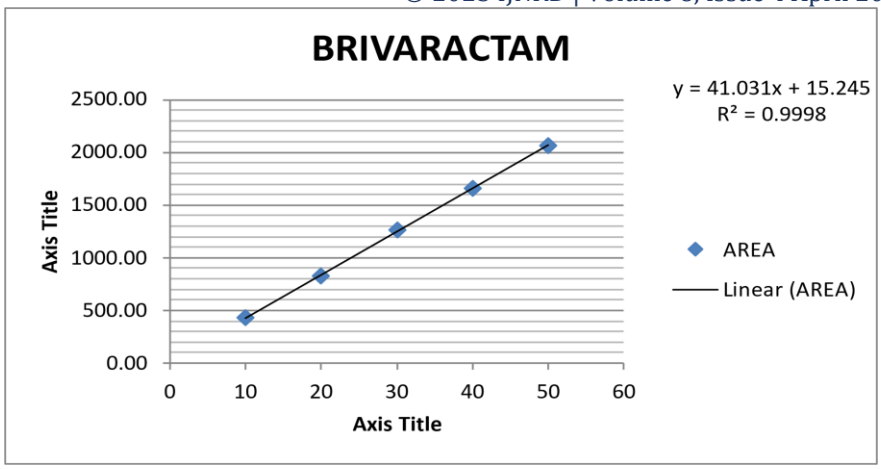



**Fig. No. 3** Calibration curve of Brivaracetam

### B. Accuracy:

As per Q2(R1), The accuracy for assay of a drug substance can be studied from 80 to 120% of the test solution.

**Table No. 6 : Accuracy results**

| Method | Drug | Level (%) | Amt. taken (ug/ml) | Amt. taken (ug/ml) | Absorbance (Mean* ± S.D.) | Amt. recovered (Mean *±S.D.) | %Recovery (Mean *± S.D.) |
|---|---|---|---|---|---|---|---|
| RP-HPLC METHOD | BRIV | 80% | 10 | 8 | 17.76±0.045 | 7.76±0.045 | 97.00±0.56 |
| | | 100% | 10 | 10 | 19.75±0.006 | 9.75±0.006 | 97.55±0.06 |
| | | 120% | 10 | 12 | 21.79±0.012 | 11.79±0.012 | 98.30±0.10 |

% Recovery = 100.037%, Acceptance Criteria 98%- 102%

### C. Precision

**Table 7: Precision results**

**Intra-day Precision:**

| Concentration(µg/ml) | Area | %Amt Found | % RSD |
|---|---|---|---|
| 10 | 424.05 | 99.61 | 1.1942 |
| 30 | 1264.38 | 101.11 | 0.1777 |
| 50 | 2858.88 | 99.59 | 0.1582 |

**Interday Precision:**

| Concentration(µg/ml) | Area | %Amt Found | % RSD |
|---|---|---|---|
| 10 | 407.91 | 99.42 | 0.33 |
| 30 | 1244.55 | 101.11 | 0.04 |
| 50 | 2044.54 | 99.69 | 0.06 |

Acceptance Criteria: Assay Value= 90-110%, % RSD=not more than 2

**D. Robustness:**

**Table No. 8 : Robustness results**

| Parameters | Conc. (µg/ml) | Amt. of detected (mean± SD) | % RSD |
|---|---|---|---|
| **Chromatogram of flow change 0.6 ml** | 40 | 1942.34 ± 1.54 | 0.13 |
| **Chromatogram of flow change 0.8 ml** | 40 | 1449.40±0.00 | 0.00 |
| **Chromatogram of comp change wavelength change 278 nm** | 40 | 725.1±0.24 | 0.03 |
| **Chromatogram of comp change wavelength change 280 nm** | 40 | 1224.4±0.73 | 0.06 |
| **A Chromatogram of mobile phase change 44+56 ml** | 40 | 1660.5±2.00 | 0.12 |
| **Chromatogram of mobile phase change 46+54 ml** | 40 | 1663.50±4.19 | 0.25 |

**E. Repeatability**

**Table No. 9: Repeatability results**

| Concentration(µg/ml) | Area | %Amt Found | % RSD |
|---|---|---|---|
| 30 | 1248.14 | 101.40 | 0.04 |

**F. LOD and LOQ**

Standard deviation (σ) = 1.43, S = 41.03(Slope)

**LOD** = 3.3 X Avg.SD/ Slope **LOQ** = 10 X Avg.SD/Slope

**LOD = 0.115034** **LOQ = 0.34587**

1. **Slope- 41.03**
2. **Intercept- 15.24**
3. **Regression-0.999**

**Table No.10 Summery of validation Parameters by RP- HPLC**

| Parameters | Data for Brivaracetam |
|---|---|
| Linearity range | 10-50 µg/ml |
| Line of regression | y = 41.031x + 15.245 |
| Correlations coefficient | $R^2$ = 0.9999 |
| Retention time (Rf value) | 4.781 min |
| Limit of detection (µg/ml) | 0.11 |
| Limit of Quantification (µg/ml) | 0.34 |
| Accuracy (%mean recovery) | 98 |
| Intra-day precision | 0.17 |
| Inter-day precision | 0.04 |
| Robustness | Robust |
| Ruggedness | % RSD is less than 2 |

## Conclusion:

In supposition, we successfully developed and validated a novel, simple, fast, and extreme sensitivity HPLC method of estimating BRV in human plasma according to ICH guidelines in terms of linearity, precision, accuracy, robustness and repeatability. The present method is faster, highly sensitive and simpler than previously published analytical reports for brivaracetam in human plasma samples. The simple extraction procedure with Acetonitrile gave a high recovery for Brivaracetam. The analyte was stable at the various conditions of stability evaluated. All validation parameters were found to be within the allowed limit. It was found that the proposed methods were linear, accurate, reproducible, repeatable, precise, cost effective and specific providing the reliability of the methods. The developed methods are recommended for routine and quality control analysis of Brivaracetam in pharmaceutical formulation

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