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Validation of an HPLC Method for Accurate Quantification of Impurities in Brivaracetam to Ensure Drug Quality and Safety

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Abstract:

Brivaracetam (BRV) represents a significant advancement in the treatment of epilepsy, offering improved efficacy and safety over traditional antiepileptic drugs (AEDs). Despite these benefits, the presence of impurities in BRV formulations can impact drug quality and patient safety. This study presents the development and validation of a high-performance liquid chromatography (HPLC) method for the accurate quantification of key impurities in BRV. Specifically, the method targets impurities such as (R)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl)butanamide and (S)-2-((S)-2-oxo-4-propylpyrrolidin-1-yl)butanamide. The method's robustness and sensitivity are validated in accordance with International Council for Harmonization (ICH) guidelines. This analytical approach ensures the high purity of BRV, contributing to its efficacy and safety profile and supporting its continued clinical use.

Keywords Brivaracetam, Epilepsy, Antiepileptic drugs (AEDs),High-performance liquid chromatography (HPLC),Impurity analysis ,Analytical method validation, ICH guidelines.

1. Introduction:

Epilepsy is defined as a condition of the central nervous system (CNS) prone to seizure activity, affecting 70 million people worldwide [1]. Contemporary antiepileptic drugs (AEDs) are effective in treating many forms of epilepsy; nonetheless, a third of patients do not respond to treatment intervention. In addition, side effects from the current AEDs affect about half of the patients undergoing treatment [2]. Brivaracetam (BRV), an AED that was just licensed, binds to synaptic vesicle protein 2A (SV2A) with great selectivity and affinity. Though SV2A is widely distributed throughout the central nervous system, it is believed to have a part in regulating synaptic vesicle exocytosis and neurotransmitter release. Levetiracetam (LEV) and BRV are comparable chemically, BRV, on the other hand, demonstrates stronger selectivity, a higher affinity for the

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SV2A binding site, and more selectivity . BRV is more potent and effective in animal models of epilepsy [3].

Brivaracetam, chemically known as [(S)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl)butanamide], is a highly potent anticonvulsant employed in the management of partial-onset seizures in epilepsy patients. Its mechanism of action involves binding to the synaptic vesicle protein 2A (SV2A), a crucial modulator of neurotransmitter release, which stabilizes neuronal activity and helps in preventing seizures. Available in various formulations, including tablets, oral solutions, and injectables, brivaracetam is rapidly absorbed, reaching peak plasma concentrations approximately one hour after oral administration. Its molecular formula is C11H20N2O2, with a molecular weight of 212.29 g/mol. The drug undergoes hepatic metabolism primarily through hydrolysis and hydroxylation, with renal excretion contributing to an elimination half-life of 8-9 hours.

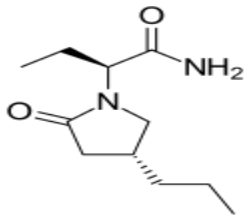

Fig No.1. Structure of Brivaracetam

The chemical synthesis of brivaracetam, however, can introduce isomeric impurities, such as (R)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl)butanamide and (S)-2-((S)-2-oxo-4-propylpyrrolidin-1-yl)butanamide, along with related substances like (S)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl)butanoic acid and (S)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl)butane nitrile(4). The analysis of related substances in brivaracetam formulations by HPLC with specific acceptance criteria for each impurity. The impurity BRV-SS should not exceed 1.0% area, while BRV-RR and BRV-RS should each not exceed 0.15% area. These stringent criteria ensure the purity of brivaracetam, minimizing potential adverse effects and maintaining its efficacy and safety for clinical use. These impurities, which can result from synthesis or degradation processes, are critical to identify and quantify due to their potential impact on the drug's efficacy and safety, potentially causing adverse effects ranging from mild somnolence to severe psychiatric symptoms(5).

To ensure the quality and safety of brivaracetam formulations for clinical use, this study aims to validate a high-performance liquid chromatography (HPLC) method for the accurate

quantification of these impurities. Adhering to International Council for Harmonization (ICH) guidelines, this validation process will ensure that brivaracetam products meet stringent quality standards, ultimately safeguarding patient health.

2. Materials and Method:

2.1. Materials Used:

The study follows Standard Test Procedure (STP) No. AFPSTP-008, Revision No. 03, utilizing high-performance liquid chromatography (HPLC) equipped with a UV/PDA detector and appropriate software. The reagents and solvents used include methanol (HPLC grade, Spectrochem or equivalent), acetonitrile (HPLC grade, Merck or equivalent), and trifluoroacetic acid (HPLC grade, Merck or equivalent). The mobile phase is prepared by mixing methanol, acetonitrile, and trifluoroacetic acid in a ratio of 90:10:0.1 (v/v), filtering through a 0.45 µm membrane filter, and sonication to degas. The diluent consists of methanol and acetonitrile in a 90:10 (v/v) ratio, which is also used for needle wash. The seal wash solution is composed of water and acetonitrile in a 90:10 (v/v) ratio. Chromatographic separation is achieved using a Chiralpak IG column (250 x 4.6 mm, 5 µm, Part No: 87325A). The detector wavelength is set at 210 nm, with an injection volume of 15.0 µL and a flow rate of 0.50 mL/min. The column temperature is maintained at 30°C, and the sample temperature is set at 25°C. The total run time for the analysis is 30 minutes.

2.2. Method:

2.2.1.Standards Used:

The study utilized various compounds for the analysis of related substances in brivaracetam formulations. The primary active pharmaceutical ingredient (API), (S)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl)butanamide (BRV-API), was obtained from TCG Life sciences Pvt. Ltd., batch number CR494-15468-78-BRV, with a potency of 99.8% and Additional impurities and related substances included (S)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl) butanoic acid (BRV-IMP-1) from batch CR494-16260-36-BRV-I-ACID-Imp, with a potency of 95.4%, and (S)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl) butanenitrile (BRV-IMP-2) from batch CR494-15882-61-BRV Im-3-P, with a potency of 99.5% and Other compounds included (S)-N-((S)-1Amino-1-Oxobutan-2-yl)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl)butanamide (BRV-IMP-3), batch CR494-16260-37-BRV-diam-P, with a potency of 93.6% and (3R)-N-((1S)-1-Amino-1-oxobutan-2-yl)-3-(Hydroxymethyl)Hexanamide (BRV-I), batch CR494-16471-I-BRV-I, with a potency of 99.0%

and (3R)-N-((1S)-1-Amino-1-oxobutan-2-yl)-3-(chloromethyl) hexanamide (BRV-II), batch CR494-16260-57-BRV-II-F, with a potency of 98.4%.Additionally, (R)-2-((S)-2-oxo-4-propylpyrrolidin-1-yl) butanamide (BRV-RS) from batch CR494-16042-89-BRV-API-RS-Imp, with a potency of 98.0% and, (R)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl) butanamide (BRV-RR) from batch CR494-15882-94-BRV-III-RR-P, with a potency of 97.4% and (S)-2-((S)-2-oxo-4-propylpyrrolidin-1-yl) butanamide (BRV-SS) from batch CR494-15883-79-BRV-III-SS-P, with a potency of 97.7% were also included.

2.2.2. Sample:

The primary active pharmaceutical ingredient, (S)-2-((R)-2-oxo-4-propylpyrrolidin-1-yl) butanamide (brivaracetam), used in this study was obtained from TCG Life sciences Pvt. Ltd. The specific batch number for this compound is CR494-16260-66-BRV, and it was manufactured in May 2021

Preparation of standard solution:

Weigh about 10 mg of BRV standard and transfer in a 10 mL volumetric flask. Add 8 mL of diluents and sonicate to dissolve completely and make up the volume up to the mark with diluent and mix well. *[Concentration: BRV: 1.0 mg/mL]*

Preparation of Sample solution:

Weigh about 50 mg of BRV sample and transfer in a 50 mL volumetric flask. Add about 40 mL of diluents and sonicate to dissolve completely and make up the volume up to the mark with diluents and mix well.

Procedure:

After equilibrating the column, separately inject diluents as blank and standard solution. If the system suitability criteria pass then inject sample solutions as per the sequence given below.

Table-1: Injection sequence

S. No.	Solution details	No. of Injections
	Blank (diluent) solution	1 (at least)
	Standard solution	1
	Blank (diluent) solution	1 (at least)
	Sample solution-1	1
	Sample solution-2	1

	Standard solution bracketing	1

2.2.3EXPERIMENTAL PLAN AND DATA EVALUATION:

The analytical method validation will be executed as per the following plan:

The experiments may be done as sequential or simultaneous operations.

Sample sequence of each experiment may be run together or independently with necessary alteration of sample sequence.

The system suitability parameters should be monitored throughout the validation study.

Pre evaluation sequence shall be conducted by injecting blank standard preparation, system suitability preparation etc. (If applicable).

Unless otherwise specified in test method, bracketing standard to be injected after every six sample injections or at the end of the sequence, whichever is earlier.

There is no restriction for number of blank injection.

1.Specificity:

Experiment:

Prepare and analyze the blank solution, standard solution and sample solution as per analytical method.

Prepare selectivity solutions and spiked sample solution as below:

Table-2: Selectivity solution

S. No.	**Sample Name**	**Weight taken (mg)**	**Diluted to mL (Stock-1)**	**Pipette volume from stock-1**	**Diluted to mL (Stock-2)**	**Pipette volume from stock-2**	**Diluted to mL**	**Final conc. (in ppm)**	**% w/w w.r.t sample conc.**
1.	BRV-IMP-1	5.0	20	3.0	50	5.0	50	1.5	0.15
2.	BRV-IMP-2	5.0	20	3.0	50	5.0	50	1.5	0.15
3.	BRV-IMP-3	5.0	20	3.0	50	5.0	50	1.5	0.15
4.	BRV-I	5.0	20	3.0	50	5.0	50	1.5	0.15

5.	BRV-SS	10.0	10	5.0	50	5.0	50	10.0	1.0
6.	BRV-RR	5.0	20	3.0	50	5.0	50	1.5	0.15
7.	BRV-RS	5.0	20	3.0	50	5.0	50	1.5	0.15
8.	BRV-II	5.0	200	2.5	250	1.5	50	7.5[#]	0.00075

[#] **Final concentration for BRV-II has been calculated in µg/g unlike other impurities in µg/mL.**

Preparation of Spiked sample solution:

Weigh about 50 mg of BRV sample and transfer in a 50 mL volumetric flask. Add 5.0 mL each of Selectivity stock-2 solution of BRV-IMP-1, BRV-IMP-2, BRV-IMP-3, BRV-I, BRV-SS, BRV-RR, BRV-RS, and 1.5 mL of selectivity stock-2 of BRV-II into the same volumetric flask and sonicate to dissolve completely. Make up the volume up to the mark with diluent and mix well *[Concentration is about 1000 ppm of BRV and 1.5 ppm of BRV-IMP-1, BRV-IMP-2, BRV-IMP-3, BRV-I, BRV-RR, BRV-RS, 10.0 ppm of BRV-SS and 7.5 µg/g(ppm) of BRV-II]*

2.LOD and LOQ:

Determination of LOD and LOQ:

To prepare the LOD-LOQ solutions, start by weighing about 10 mg each of BRV and BRV-SS standards in separate 10 mL volumetric flasks. Add about 8 mL of diluent, sonicate to dissolve, then dilute to the mark with diluents (each about 1000 ppm). For BRV-RR and BRV-RS standards, weigh about 5 mg each in separate 20 mL volumetric flasks, add about 10 mL of diluents, sonicate to dissolve, then dilute to the mark with diluents (each about 250 ppm). Combine 0.5 mL each of BRV and BRV-SS stock solutions with 2.0 mL each of BRV-RR and BRV-RS stock solutions in a 20 mL volumetric flask, dilute to the mark with diluents (about 25 ppm each). Finally, pipette 1.0 mL of this mixture into a 50 mL volumetric flask, dilute to the mark with diluents, achieving a final concentration of about 0.5 ppm for BRV, BRV-SS, BRV-RR, and BRV-RS.

2.2. LOQ Precision:

Experiment:

Prepare and analyze the blank solution and standard solution as per analytical method.

2.3.LOD Verification:

Experiment:

Prepare and analyze the blank solution and standard solution as per analytical method.

Prepare a solution containing the analytes at LOD Level concentration from above experiment and analyze LOD level concentration thrice as per the analytical method.

Preparation of LOD Level solution:

Pipette out 3.0 mL of LOQ Level solution into a 10 mL volumetric flask and make the volume up to the mark with diluents and mix well.

3.Linearity:

Experiment:

Prepare and analyze the blank solution and standard solution as per analytical method.

Prepare a series of BRV preparations over a range starting from 50 to 150% of nominal sample concentration (i.e. 1000 ppm); another series of specified impurities concentrations ranging from LOQ to 200% of specification limit and BRV for single maximum unknown impurity.

Preparation of linearity solution from LOQ to 200% of the specification limit for each specified impurities and single maximum unknown impurity:

To prepare the linearity stock solutions, begin by weighing about 10 mg each of BRV and BRV-SS standards in separate 10 mL volumetric flasks. Add about 8 mL of diluent, sonicate to dissolve, then dilute to the mark with diluents (each about 1000 ppm). For BRV-RR and BRV-RS standards, weigh about 5 mg each in separate 20 mL volumetric flasks, add about 10 mL of diluents, sonicate to dissolve, then dilute to the mark with diluents (each about 250 ppm). Combine 5.0 mL each of BRV and BRV-SS stock solutions with 3.0 mL each of BRV-RR and BRV-RS stock solutions in a 50 mL volumetric flask, dilute to the mark with diluents, achieving final concentrations of about 100 ppm each for BRV and BRV-SS, and about 15 ppm each for BRV-RR and BRV-RS.

Preparation of linearity solution from 50% to 150% of sample concentration (i.e. 1000 ppm) of BRV:

Weigh about 50 mg of BRV standard in a 20 mL volumetric flask. Add about 15 mL of diluent and sonicate to dissolve. Dilute up to the mark with diluents and mix well *(Concentration is about 2500 ppm of BRV).*

4.Accuracy (% Recovery):

Experiment:

Prepare and analyze the Blank (Diluents) and standard solution as per analytical method.

The accuracy is to be determined by injecting the solutions containing analytes ranging from LOQ to 200% of specified limit concentration of specified impurities of BRV.

To prepare the accuracy stock solutions, weigh about 10 mg each of BRV and BRV-SS standards in separate 10 mL volumetric flasks, add about 8 mL of diluents, sonicate to dissolve, then dilute to the mark with diluents (each about 1000 ppm). For BRV-RR and BRV-RS standards, weigh about 5 mg each in separate 20 mL volumetric flasks, add about 10 mL of diluents, sonicate to dissolve, then dilute to the mark with diluent (each about 250 ppm). Combine 5.0 mL of BRV-SS stock solution, 3.0 mL each of BRV-RR and BRV-RS stock solutions in a 50 mL volumetric flask, dilute to the mark with diluents (about 100 ppm BRV-SS, 15 ppm BRV-RR, and 15 ppm BRV-RS). For another mixture, combine 0.5 mL of BRV-SS stock solution, 2.0 mL each of BRV-RR and BRV-RS stock solutions in a 20 mL volumetric flask, dilute to the mark with diluents (about 25 ppm each of BRV-SS, BRV-RR, and BRV-RS).

Preparation of Accuracy standard solution:

Further pipette out 5.0 mL each of Accuracy stock solution-5and 0.5 mL of Accuracy stock solution-1 transfer into a 50 mL volumetric flask. Make up the volume up to the mark with diluents and mix well *(Concentration is about 10 ppm each of BRV, BRV-SS and 1.5 ppm of each of BRV-RR and BRV-RS).*

Preparation of Accuracy samples:

Prepare the sample for accuracy as unspiked sample and spiked at LOQ Level, 100% and 200 % level of specification limit of known impurities in triplicate.

Preparation of unspiked sample solution: (3 preparations)

Weigh accurately about 50 mg of BRV sample and transfer in a 50 mL volumetric flask. Add about 40 mL of diluent and sonicate to dissolve completely and make up the volume up to the mark with diluent and mix well. *[Concentration is about 1000 ppm of BRV)*

Accuracy – LOQ: (3 preparations)

Weigh accurately about 50 mg of BRV sample and transfer in a 50 mL volumetric flask. Add about 40 mL of diluent and sonicate to dissolve completely. Then add 1.0 mL of Accuracy stock solution-6 and make up the volume up to the mark with diluents and mix well. *[Concentration is about 1000 ppm of BRV and 0.5 ppm of each of BRV-SS, BRV-RR, BRV-RS]*

Accuracy – 100%: (3 preparations)

Weigh accurately about 50 mg of BRV sample and transfer in a 50 mL volumetric flask. Add about 40 mL of diluents and sonicate to dissolve completely. Then add 5.0 mL of Accuracy stock solution-5 and make up the volume up to the mark with diluents and mix well. *[Concentration is about 1000 ppm of BRV and 10 ppm of BRV-SS and 1.5 ppm each of BRV-RR, BRV-RS].*

Accuracy – 200%: (3 preparations)

Weigh accurately about 50 mg of BRV sample and transfer in a 50 mL volumetric flask. Add about 30 mL of diluents and sonicate to dissolve completely. Then add 10.0 mL of Accuracy stock solution-5 and make up the volume up to the mark with diluents and mix well. *[Concentration is about 1000 ppm of BRV and 20 ppm of BRV-SS and 3 ppm each of BRV-RR, BRV-RS].*

5.Precision:

5.1.Method Precision :

Experiment :

Prepare and analyze the blank solution, standard solution and sample solution (6 preparations) as per the analytical method.

5.2.Intermediate Precision:

Experiment:

Intermediate precision express within laboratory precision by different analysts, different instruments, and different columns of same make with different serial numbers by using the same sample and method as described in method precision on a different day as per the following matrix.

Prepare and analyze the blank solution, standard solution and sample solution (6 preparations) as per the analytical method.

6.Range:

Experiment:

The range shall be determined from Linearity, Precision and Accuracy study for related substances .

7.Stability of analytical solution:

Experiment:

Prepare blank solution, standard solution and sample solution as per the analytical method.

Analyze the Blank, Sample Solution initially, after 12 hours, after 24 hours and after 48 hours at room temperature.

8.Robustness:

Experiment:

Prepare and analyze the blank solution, standard solution and sample solution (duplicate preparations) as per the analytical method and inject using different chromatographic conditions as shown below:

Change in Flow rate (± 0.1 mL).

Change in detection wavelength (±2 nm).

Change in volume of Trifluoroacetic acid (± 10%) in Mobile phase.

Change in volume of Acetonitrile (± 10%) in Mobile phase.

Change in column oven temperature (± 2°C).

3. Results and Discussion:

1. Specificity:

- No interfering peak was observed at the retention time of BRV and its specified impurity peak in blank.
- The peak due to BRV and its isomeric impurities were well resolved from each other and also from other specified impurities and any other peaks (Refer table 3).
- Purity angle found to be less than purity threshold for BRV and its specified impurities (Refer table 3).

Table 3: Selectivity data (Purity angle and Purity threshold)

Sample name	Purity angle and Purity threshold							
	BRV		BRV-SS		BRV-RR		BRV-RS	
	Purity angle	Purity thresh	Purity angle	Purity threshol	Purity angle	Purity threshol	Purity angle	Purity thresho
Standard[1]	0.198	0.269	NA	NA	NA	NA	NA	NA
Unspiked sample	NA	NA	4.809	6.914	NA	NA	NA	NA
Spiked sample	NA	NA	0.792	1.259	4.416	6.936	3.761	5.608
Diluted Unspiked sample	0.175	0.269	NA	NA	NA	NA	NA	NA
Diluted Spiked sample	0.187	0.267	NA	NA	NA	NA	NA	NA
BRV-SS selectivity solution	NA	NA	0.809	1.366	NA	NA	NA	NA
BRV-RR selectivity solution	NA	NA	NA	NA	4.995	6.568	NA	NA
BRV-RS selectivity solution	NA	NA	NA	NA	NA	NA	3.077	5.765

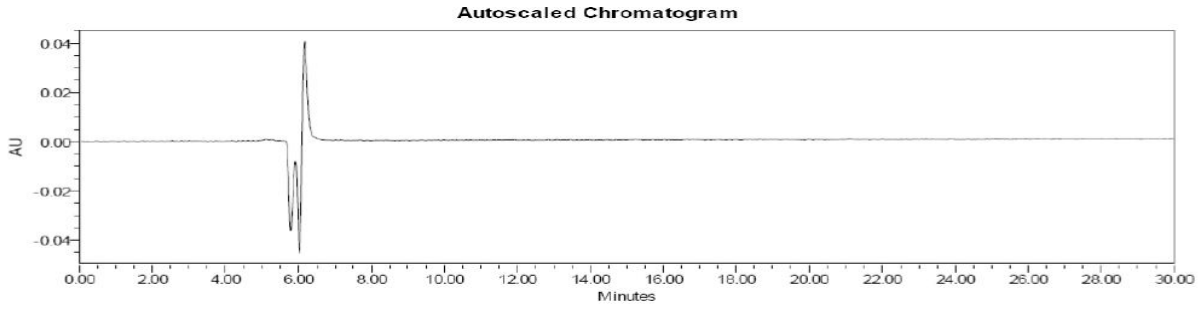

Figure 2: Specificity – Diluent (Blank)

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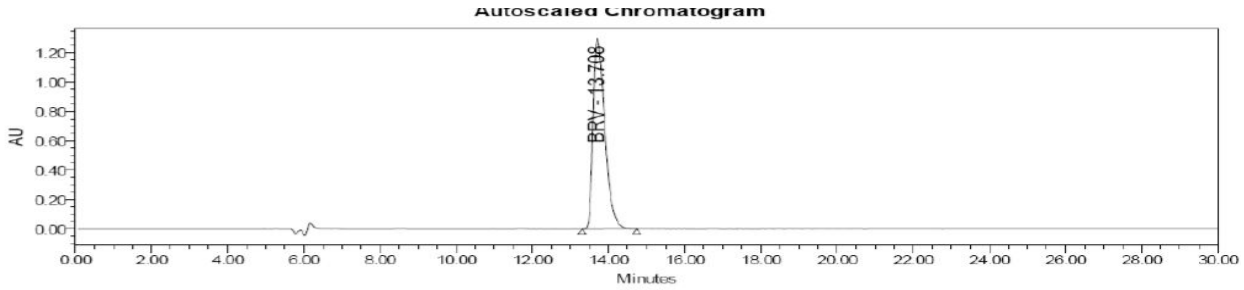

Figure 3: Specificity – Standard solution

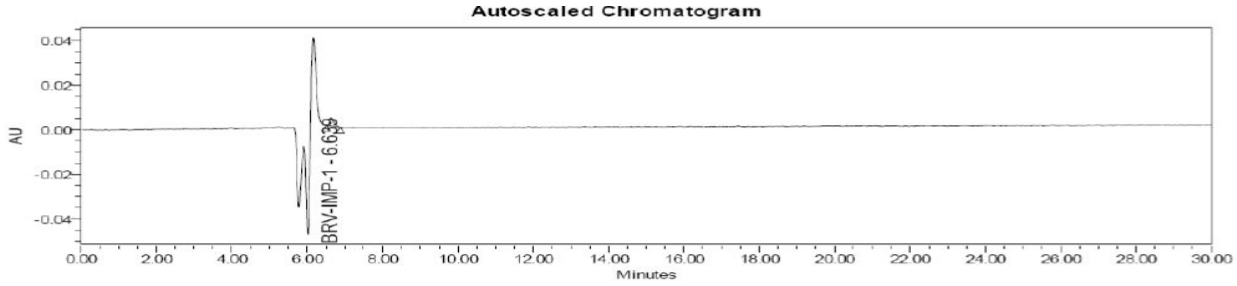

Figure 4: Specificity – BRV-IMP-1 selectivity solution

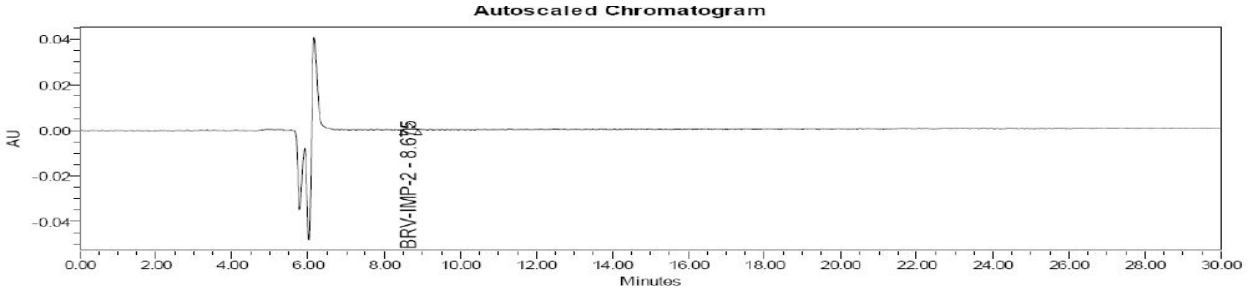

Figure 5: Specificity – BRV-IMP-2 selectivity solution

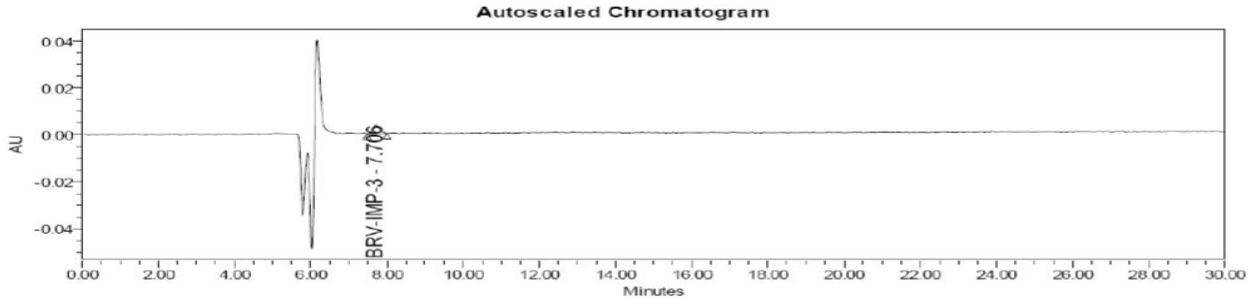

Figure 6: Specificity – BRV-IMP-3 selectivity solution

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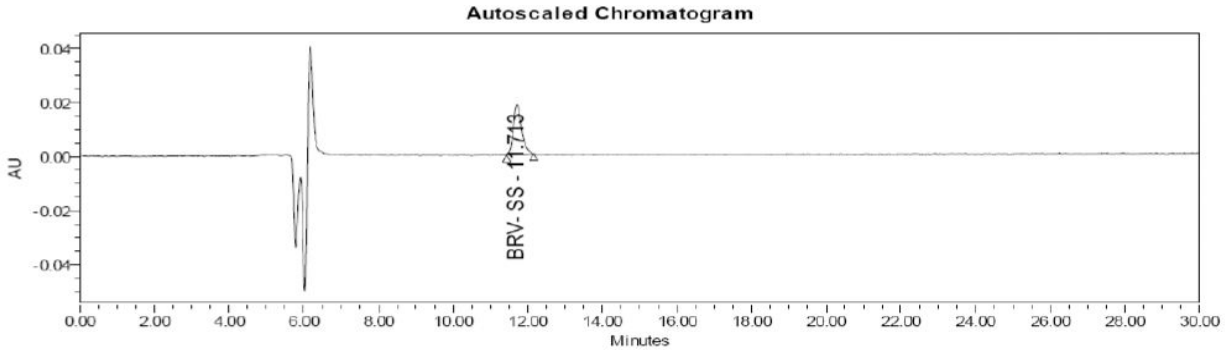

Figure 7: Specificity – BRV-SS selectivity solution

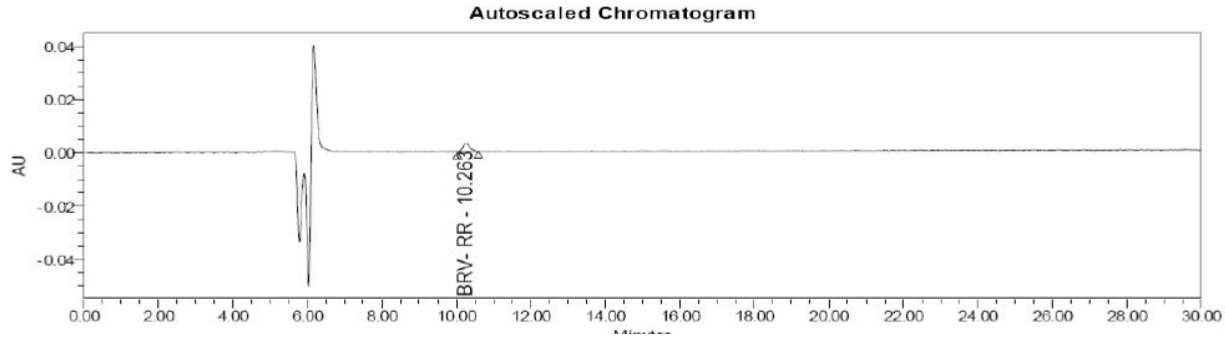

Figure 8: Specificity – BRV-RR selectivity solution

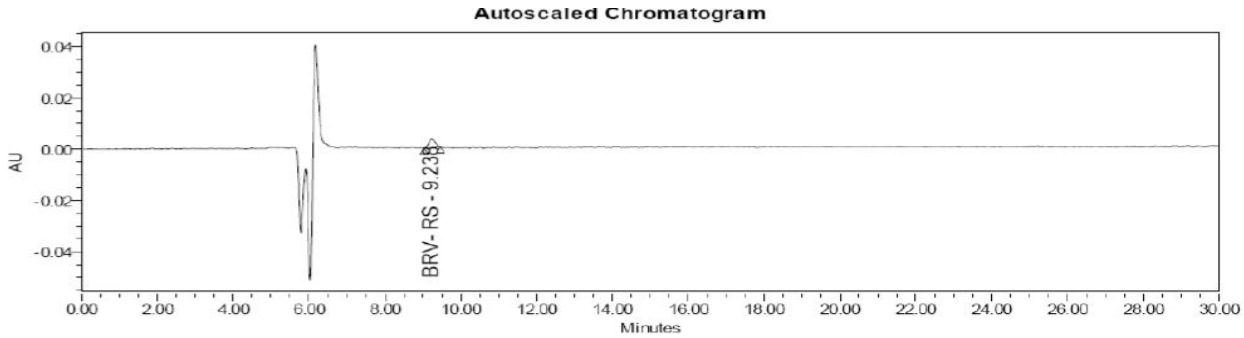

Figure 9: Specificity – BRV-RS selectivity solution

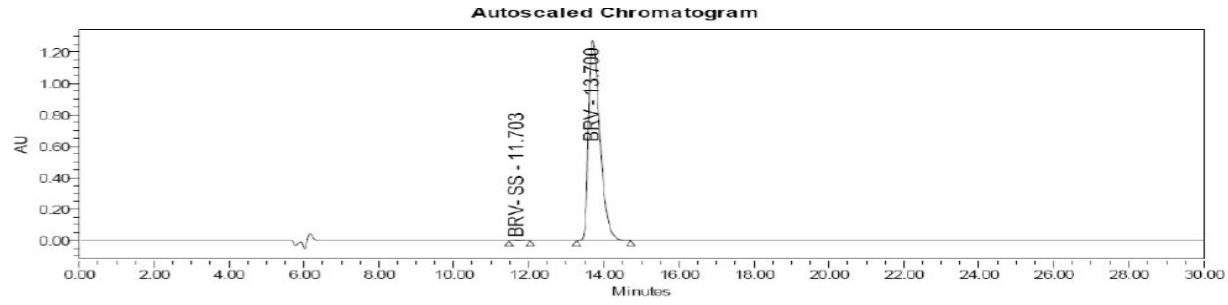

Figure 10: Specificity – Unspiked sample solution

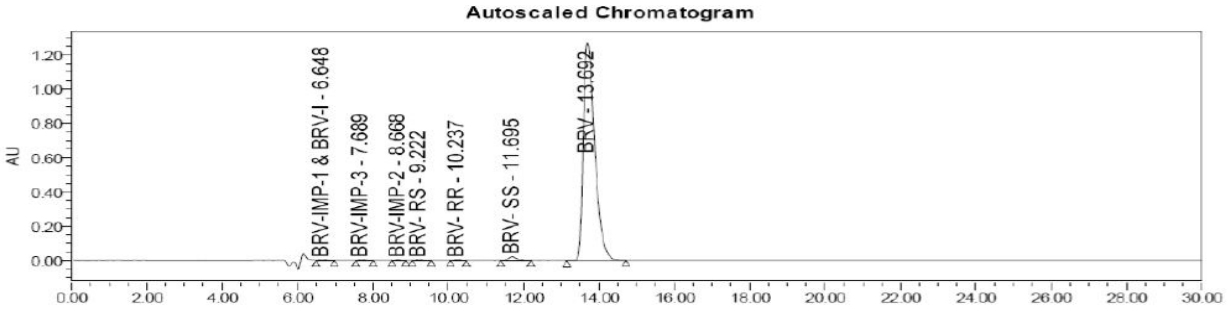

Figure 11: Specificity – Spiked sample solution

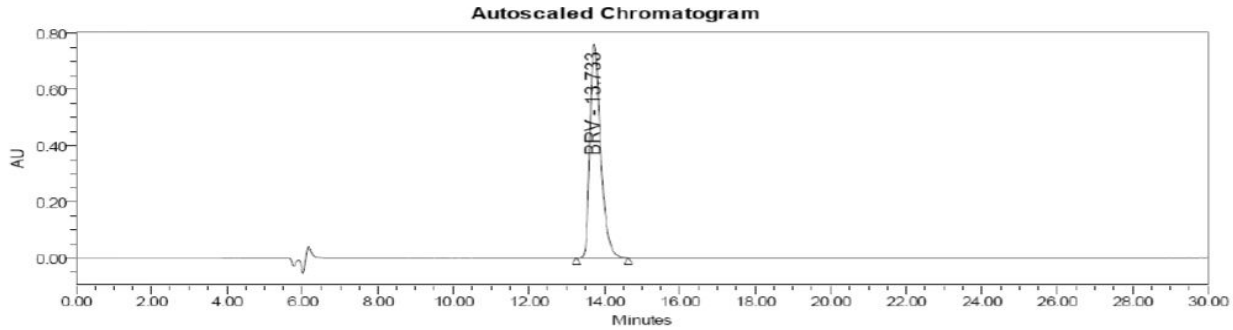

Figure 12: Specificity – Diluted standard solution

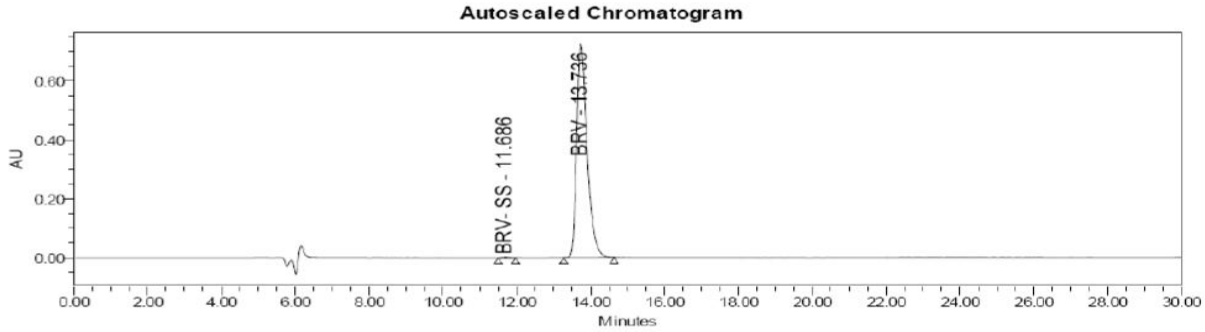

Figure 13: Specificity – Diluted unspiked sample solution

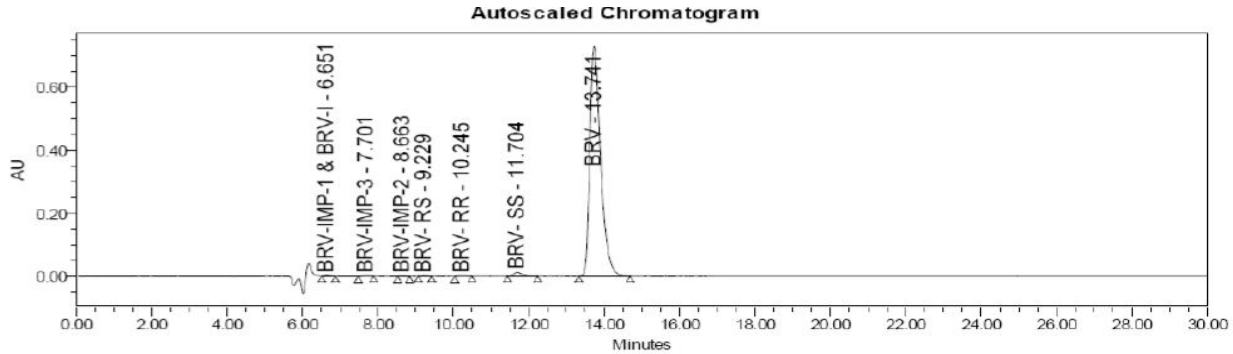

Figure 14: Specificity – Diluted Spiked sample solution

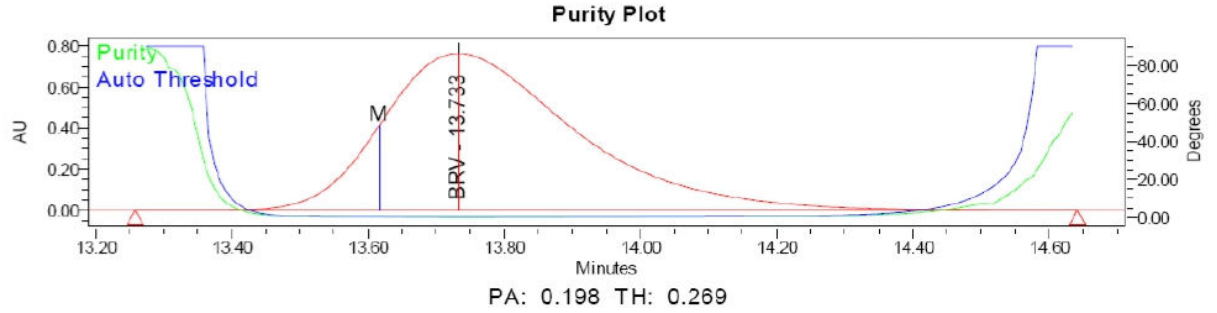

Figure 15: Specificity – Peak purity plot of diluted standard

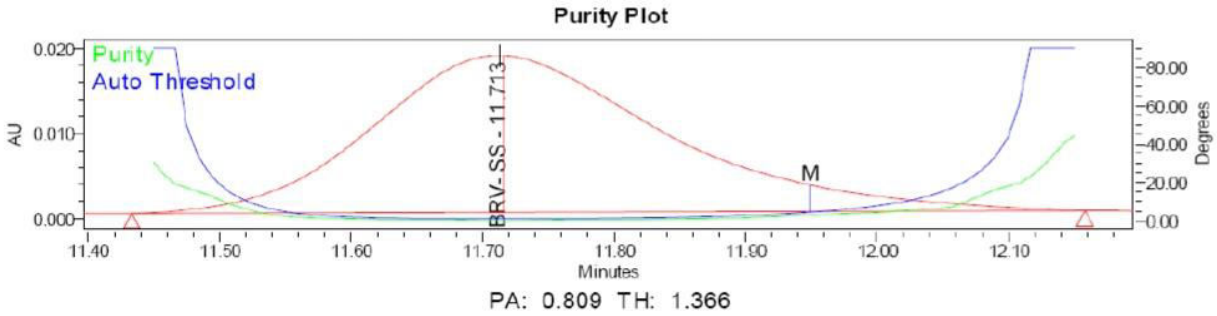

Figure 16: Specificity – Peak purity plot of BRV-SS selectivity solution

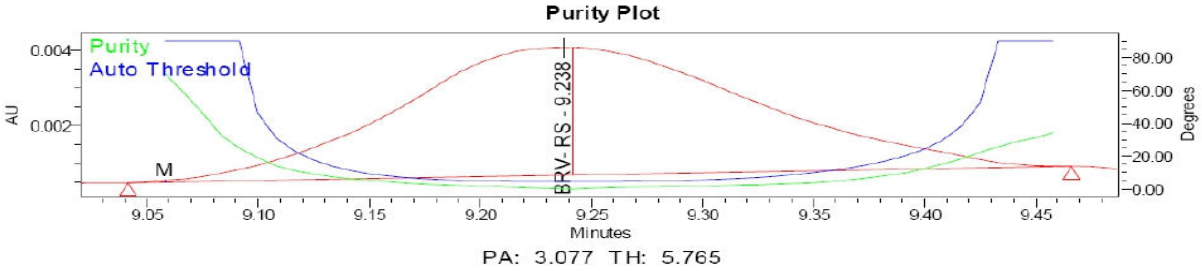

Figure 17: Specificity – Peak purity plot of BRV-RS selectivity solution

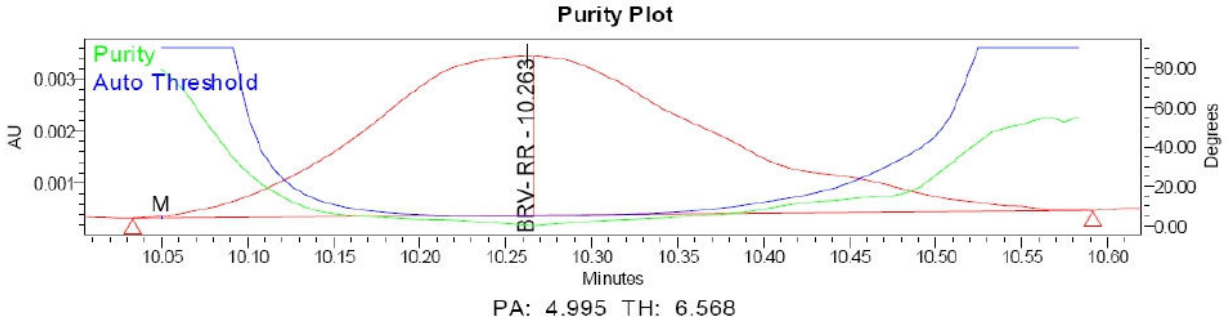

Figure 18: Selectivity – Peak purity plot of BRV-RR selectivity solution

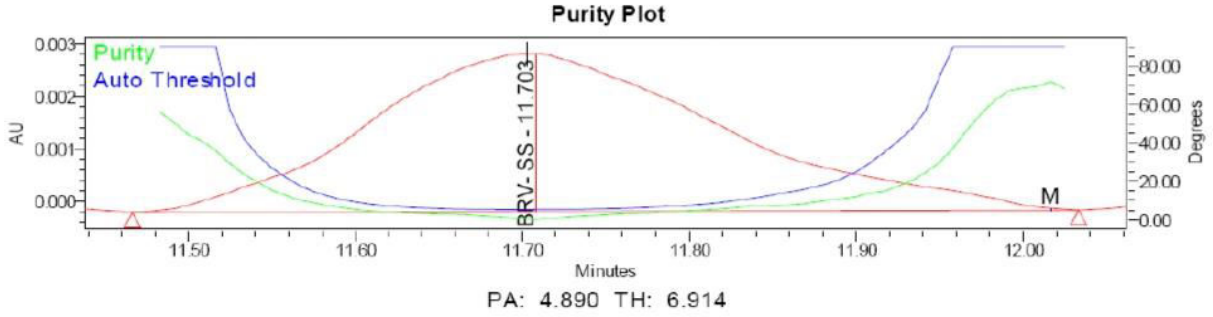

Figure 19: Specificity – Peak purity plot of Unspiked sample

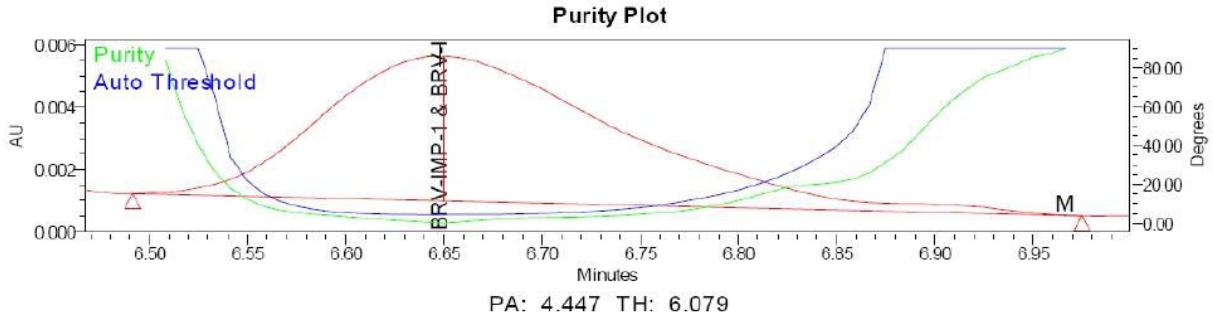

Figure 20: Specificity – Peak purity plot of Spiked sample

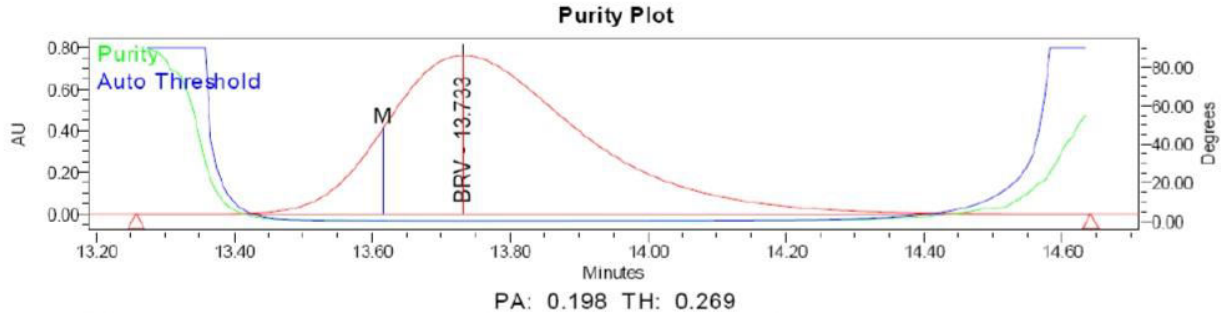

Figure 21: Specificity – Peak purity plot of Diluted standard

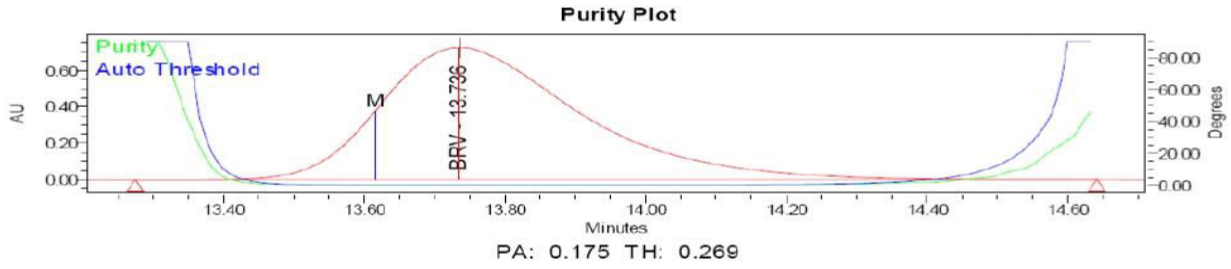

Figure 22: Specificity – Peak purity plot of Diluted Unspiked sample

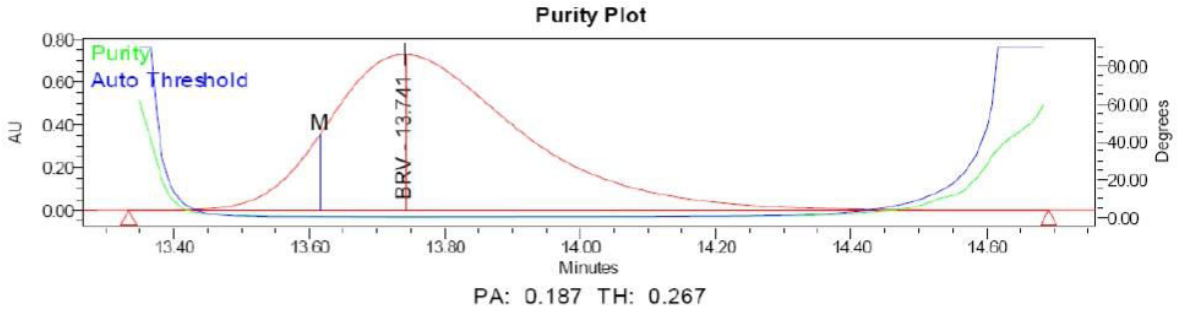

Figure 23: Specificity – Peak purity plot of Diluted Spiked sample

2. LOD and LOQ:

Table 4: LOD-LOQ Determination

Component	Area	USP S/N	Determined LOQ Conc. (%w.r.t sample concentration)	Expected LOD Conc. (%w.r.t sample concentration)
BRV	15531	134	0.05	0.02
BRV-SS	15184	79	0.05	0.02
BRV-RR	14511	100	0.05	0.02
BRV-RS	14601	187	0.05	0.01

2.2. LOQ Precision:

Table 5: Results of LOQ Precision

LOQ Level injections	BRV		BRV-SS		BRV-RR		BRV-RS	
	Area	USP S/N	Area	USP S/N	Area	USP S/N	Area	USP S/N
LOQ Conc. % w.r.t. test	0.05		0.05		0.05		0.05	
LOQ Solution, Inj.-1	15669	133	15294	78	14650	99	14532	187
LOQ Solution,	15492	134	15345	78	14262	99	14609	183

Inj.-2								
LOQ Solution, Inj.-3	15180	134	15249	76	14552	102	14564	183
LOQ Solution, Inj.-4	15633	138	15667	78	15576	105	15275	170
LOQ Solution, Inj.-5	15591	139	15099	76	15185	104	15044	166
LOQ Solution, Inj.-6	15965	141	15308	77	15316	105	15389	148
Mean	15588	NA	15327	NA	14923	NA	14902	NA
SD	**255.4030 3**		**187.2502 0**		**509.77143**		**382.8799 5**	
% RSD	**1.6**		**1.2**		**3.4**		**2.6**	

2.3. LOD Verification:

Table 6: Results of LOD verification

LOD Level injections	**BRV**		**BRV-SS**		**BRV-RR**		**BRV-RS**	
	Area	**USP S/N**	**Area**	**USP S/N**	**Area**	**USP S/N**	**Area**	**USP S/N**
LOD Conc. % w.r.t. test	**0.02**		**0.02**		**0.02**		**0.01**	
LOD Solution, Inj.-1	4836	41	4614	23	5064	32	4916	50
LOD Solution, Inj.-2	4786	41	4735	23	4368	29	4642	51
LOD Solution, Inj.-3	5102	42	4752	24	4385	31	4686	52

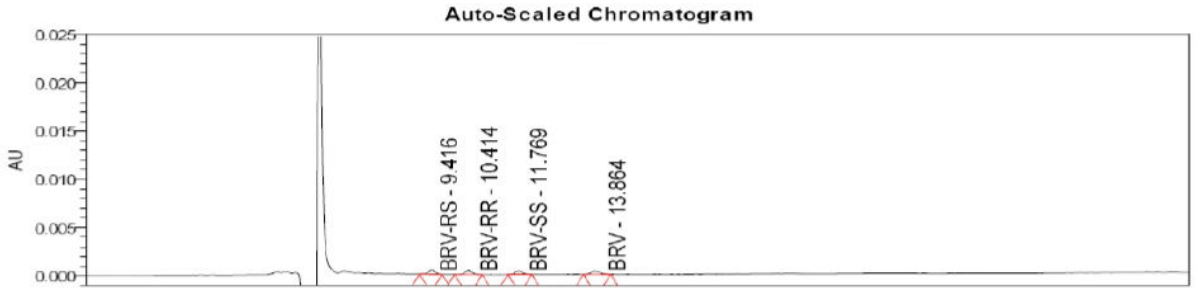

Figure 24: LOD Verification – 1st injection

3. Linearity:

Table 7A: Linearity data of BRV

(LOQ to 200% of specification limit)

Linearity Level	**Percent (%) (w.r.t spec conc.)**	**Concentration (in % w.r.t. sample concentration)**	**Mean area (n = 2)**
Level – 1	LOQ	0.051	15780
Level – 2	50	0.507	153076
Level – 3	100	1.013	305438
Level – 4	150	1.520	463218
Level – 5	200	2.027	616120
Correlation coefficient			0.9999
Y-Intercept			-710.034
Slope			304254.896
Residual standard deviation			1646.777451
Residual sum of squares			8135627.917

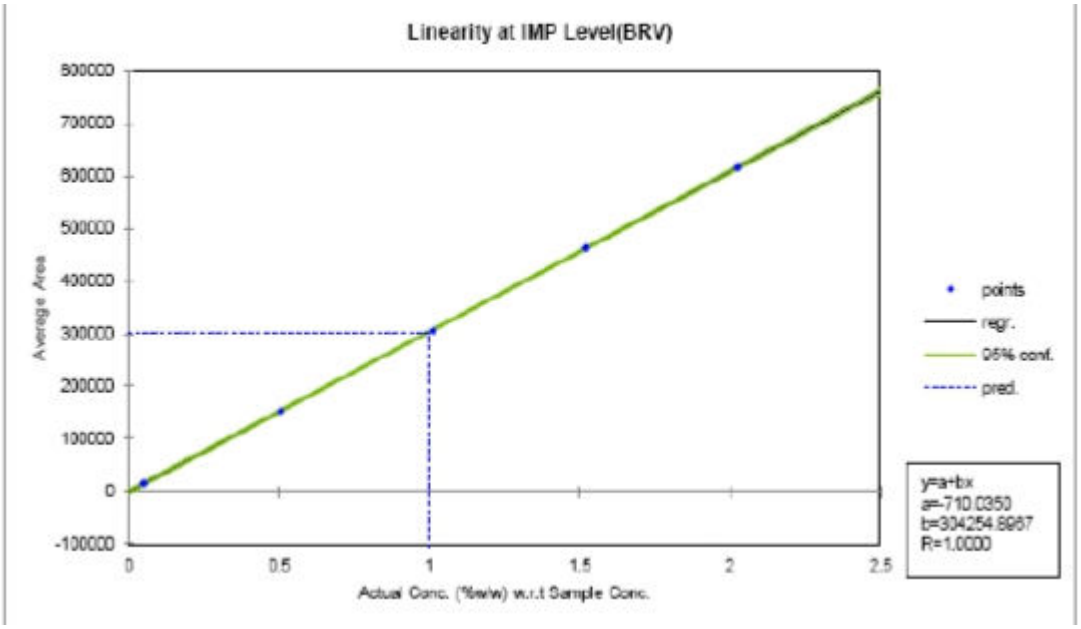

Figure 25: Linearity graph of BRV (LOQ to 200% of specification limit)

Table 7B: Linearity data of BRV-SS

(LOQ to 200% of specification limit)

Linearity Level	**Percent (%) (w.r.t spec conc.)**	**Concentration (in % w.r.t. sample concentration)**	**Mean area (n = 2)**
Level – 1	LOQ	0.050	15194
Level – 2	50	0.505	148504
Level – 3	100	1.009	294324
Level – 4	150	1.514	446274
Level – 5	200	2.018	594381
		Correlation coefficient	0.9999
		Y-Intercept	-424.208
		Slope	294466.565
		Residual standard deviation	1598.303855
		Residual sum of squares	7663725.634

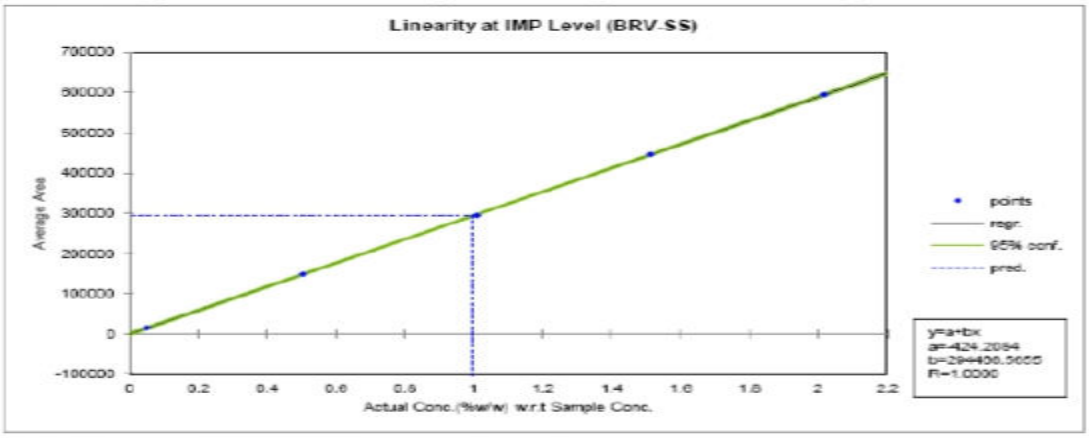

Figure 26: Linearity graph of BRV-SS (LOQ to 200% of specification limit)

Table 7C: Linearity data of BRV-RR

(LOQ to 200% of specification limit)

Linearity Level	**Percent (%) (w.r.t spec conc.)**	**Concentration (in % w.r.t. sample concentration)**	**Mean area (n = 2)**
Level – 1	LOQ	0.051	14729
Level – 2	50	0.076	21830
Level – 3	100	0.152	43068
Level – 4	150	0.228	65107
Level – 5	200	0.304	87225
		Correlation coefficient	0.9999
		Y-Intercept	12.017
		Slope	285944.986
		Residual standard deviation	324.3004016
		Residual sum of squares	315512.2514

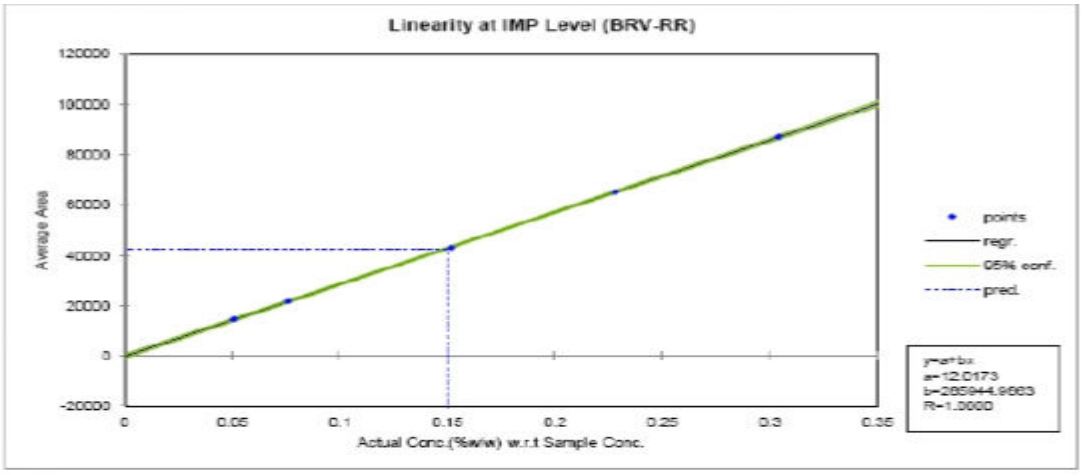

Figure 27: Linearity graph of BRV-RR (LOQ to 200% of specification limit)

Table 7D: Linearity data of BRV-RS

(LOQ to 200% of specification limit)

Linearity Level	**Percent (%) (w.r.t spec conc.)**	**Concentration (in % w.r.t. sample concentration)**	**Mean area (n = 2)**
Level – 1	LOQ	0.050	15280
Level – 2	50	0.074	22308
Level – 3	100	0.149	42849
Level – 4	150	0.223	65128
Level – 5	200	0.298	86767
		Correlation coefficient	0.9998
		Y-Intercept	685.202
		Slope	288054.384
		Residual standard deviation	497.920361
		Residual sum of squares	743774.0578

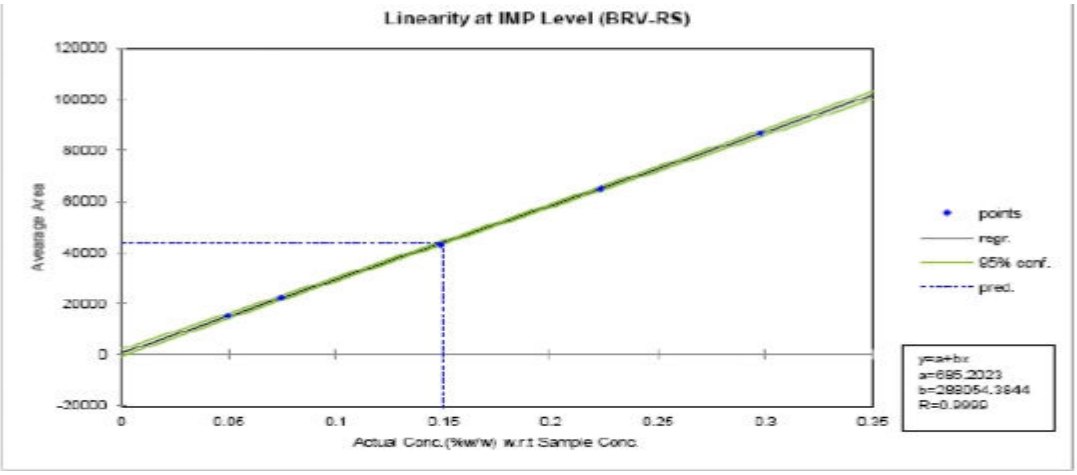

Figure 28: Linearity graph of BRV-RS(LOQ to 200% of specification limit)

Accuracy:

Mean recovery and % RSD for accuracy sample preparations were within the acceptance criteria (Refer table 8 to 9).

Table 8: % Recovery data of BRV-SS

Level No.	Test ID	Theoretical Amount (% w/w)	Amount found (% w/w)	% Recovery (Accuracy)
Recovery level-1 (LOQ)	Test-1	0.21232	0.21144	99.585
	Test-2	0.21294	0.21232	99.708
	Test-3	0.21303	0.21186	99.449
			Mean recovery	**100**
			SD	**0.129537**
			% RSD	**0.1**
Recovery level-2 (100%)	Test-1	1.16863	1.15600	98.919
	Test-2	1.17014	1.15729	98.901
	Test-3	1.16979	1.15435	98.680
			Mean recovery	**99**
			SD	**0.133332**
			% RSD	**0.1**
Recovery level-3	Test-1	2.17397	2.15075	98.932

(200%)	Test-2	2.17390	2.15928	99.327
	Test-3	2.17529	2.15930	99.265
			Mean recovery	**99**
			SD	**0.212586**
			% RSD	**0.2**

Table 9: % Recovery data of Recovery data of BRV-RR

Level No.	**Test ID**	**Theoretical Amount (% w/w)**	**Amount found (% w/w)**	**% Recovery (Accuracy)**
Recovery level-1 (LOQ)	Test-1	0.05071	0.04940	97.422
	Test-2	0.05071	0.04920	97.036
	Test-3	0.05071	0.04890	96.428
			Mean recovery	**97**
			SD	**0.501090**
			% RSD	**0.5**
Recovery level-2 (100%)	Test-1	0.15212	0.14602	95.993
	Test-2	0.15212	0.14666	96.410
	Test-3	0.15212	0.14481	95.195
			Mean recovery	**96**
			SD	**0.617390**
			% RSD	**0.6**
Recovery level-3 (200%)	Test-1	0.30424	0.29587	97.251
	Test-2	0.30424	0.29791	97.920
	Test-3	0.30424	0.29717	97.675
			Mean recovery	**98**
			SD	**0.338780**
			% RSD	**0.3**

Method Precision:

%RSD of the content of % area for the isomeric impurities and total impurities results from six sample preparations were within the acceptance criteria. (Refer table 10A & 11).

Table 10A: Method precision data for Related substances (Unspiked sample)

Sample preparations	% Content (w/w)			
	BRV-SS	**BRV-RR**	**BRV-RS**	**Total impurities**
Preparation-1	0.18035	<RL	<RL	0.18035
Preparation-2	0.18086	<RL	<RL	0.18086
Preparation-3	0.18014	<RL	<RL	0.18014
Preparation-4	0.18214	<RL	<RL	0.18214
Preparation-5	0.18332	<RL	<RL	0.18332
Preparation-6	0.18039	<RL	<RL	0.18039
Mean	**0.18**	**<RL**	**<RL**	**0.18**
Std. Deviation	**0.001264**	**NA**	**NA**	**0.001264**
%RSD	**0.7**	**NA**	**NA**	**0.7**

Table 10B: Method precision data for Related substances (Spiked sample)

Sample preparations	% Content (w/w)		
	BRV-SS	**BRV-RR**	**BRV-RS**
Preparation-1	1.27338	0.16110	0.16716
Preparation-2	1.26387	0.16042	0.16575
Preparation-	1.26597	0.15907	0.16644

3			
Preparation-4	1.27590	0.16130	0.16781
Preparation-5	1.26179	0.15997	0.16626
Preparation-6	1.27492	0.16172	0.16788
Mean	**1.3**	**0.16**	**0.17**
Std. Deviation	**0.006144**	**0.000976**	**0.000871**
%RSD	**0.5**	**0.6**	**0.5**

Table 11: % Recovery data of BRV-RS

Level No.	**Test ID**	**Theoretical Amount (% w/w)**	**Amount found (% w/w)**	**% Recovery (Accuracy)**
Recovery level-1 (LOQ)	Test-1	0.05030	0.05368	106.714
	Test-2	0.05030	0.05396	107.268
	Test-3	0.05030	0.05356	106.464
			Mean recovery	**107**
			SD	**0.411515**
			% RSD	**0.4**
Recovery level-2 (100%)	Test-1	0.15091	0.14870	98.534
	Test-2	0.15091	0.14871	98.545
	Test-3	0.15091	0.14871	98.540
			Mean recovery	**99**
			SD	**0.005668**
			% RSD	**0.0**
Recovery level-3 (200%)	Test-1	0.30182	0.29792	98.708
	Test-2	0.30182	0.29882	99.004

	Test-3	0.30182	0.29888	99.026
			Mean recovery	**99**
			SD	**0.177501**
			% RSD	**0.2**

Intermediate Precision:

1. %RSD of the content of % area for the isomeric impurities result from six sample solutions were within the acceptance criteria (Refer table 13A for unspiked sample and 13B for spiked sample).
2. %RSD of the content of % area for the isomeric impurities result from twelve spiked sample preparations (six from method precision and six from intermediate precision) were within the acceptance criteria (Refer table 12A for unspiked sample and 12B for spiked sample).

Table 12A: Intermediate precision data for Related substances (Unspiked sample) [6 preparations]

Sample preparations	% Content (w/w)			
	BRV-SS	**BRV-RR**	**BRV-RS**	**Total impurities**
Preparation-1	0.16892	<RL	<RL	0.16892
Preparation-2	0.16360	<RL	<RL	0.16360
Preparation-3	0.14735	<RL	<RL	0.14735
Preparation-4	0.17727	<RL	<RL	0.17727
Preparation-5	0.15583	<RL	<RL	0.15583
Preparation-6	0.17176	<RL	<RL	0.17176
Mean	**0.16**	**<RL**	**<RL**	**0.16**
Std.	**0.010995**	**NA**	**NA**	**0.010995**

Deviation				
%RSD	**6.7**	**NA**	**NA**	**6.7**

Table 12B: Intermediate precision data for Related substances (Spiked sample) [6 preparations]

Sample preparations	**% Content (w/w)**		
	BRV-SS	**BRV-RR**	**BRV-RS**
Preparation-1	1.26604	0.14215	0.16414
Preparation-2	1.25801	0.14359	0.16386
Preparation-3	1.26092	0.15502	0.14809
Preparation-4	1.27217	0.13244	0.14655
Preparation-5	1.21617	0.15520	0.12331
Preparation-6	1.28262	0.14312	0.15883
Mean	**1.3**	**0.15**	**0.15**
Std. Deviation	**0.022888**	**0.008665**	**0.015453**
%RSD	**1.8**	**6.0**	**10.2**

Stability of Analytical Solution:

Table 13: Stability of analytical solution: Standard preparation – at room temperature

Time points (in hours)	**USP Tailing factor**	**USP Theoretical plates**
Initial	1.4	12503
After 12 hours	1.4	12523

After 24 hours	1.4	12510
After 48 hours (48 Hours and 32 min.)	1.4	12477

Robustness:

- System suitability criteria were within the acceptance criteria.
- Overall %RSD with two results of same sample with the standard condition: n=8 (2+6) i.e. two data from changed condition and six data from method precision was as per the acceptance criteria (Refer table 14).

Table 14: Robustness [Change in flow rate (0.40 mL/minute)] for Spiked sample

Sample preparations	**% Content (w/w)**		
	BRV-SS	**BRV-RR**	**BRV-RS**
Method precision preparation-1	1.27338	0.16110	0.16716
Method precision preparation-2	1.26387	0.16042	0.16575
Method precision preparation-3	1.26597	0.15907	0.16644
Method precision preparation-4	1.27590	0.16130	0.16781
Method precision preparation-5	1.26179	0.15997	0.16626
Method precision preparation-6	1.27492	0.16172	0.16788
Robustness preparation-1	1.26735	0.16445	0.16543
Robustness preparation-2	1.26461	0.15842	0.16041
Mean	**1.3**	**0.16**	**0.17**
Std. Deviation	**0.005465**	**0.001851**	**0.002389**
%RSD	**0.4**	**1.2**	**1.4**

4. Conclusion:

The validation of the HPLC method for analyzing impurities in brivaracetam formulations confirms that the method adheres to the highest quality standards. It demonstrated specificity by effectively distinguishing brivaracetam from its impurities and other substances. The method's limits of detection (LOD) and quantitation (LOQ) are sufficiently low, ensuring accurate detection and measurement of impurities. Linearity, accuracy, and precision were all validated, showing reliable performance across a broad range of concentrations and varying conditions. Additionally, the method proved robust against changes in chromatographic parameters and exhibited stability in analytical solutions over a 48-hour period. Overall, this validated HPLC method ensures precise, accurate, and consistent impurity analysis, supporting the quality and safety of brivaracetam formulations.

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