

A Greenness Evaluated Stability-Indicating RP-HPLC Method for Simultaneous Determination of Related Substances Including Potential Azido Process Impurities in Sacubitril–Valsartan Combination.

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ABSTRACT

Sacubitril is a neprilysin inhibitor that reduces cardiac workload and improves cardiovascular function, while Valsartan is a selective angiotensin II type-1 (AT₁) receptor antagonist widely used in the management of hypertension and heart failure. In this study, a stability-indicating RP-HPLC method was developed and validated for the simultaneous determination of Sacubitril, Valsartan, and their related impurities in pharmaceutical formulations. Chromatographic separation was achieved on a Kromasil 100-5 C18 column (150 mm × 4.6 mm, 5 µm) using gradient elution at a flow rate of 1.3 mL min⁻¹ with photodiode array detection at 254 nm for Sacubitril and 225 nm for Valsartan. The method provided complete separation of both active pharmaceutical ingredients and all known impurities within an 85 min run time. Validation was performed according to ICH guidelines, demonstrating excellent specificity, precision, accuracy, linearity, sensitivity, and robustness. Recovery values ranged from 85.0% to 115.0%, and no interference from excipients or impurities was observed. Relative response factors (RRFs), limits of detection (LOD), and limits of quantification (LOQ) were established for impurity quantification. Forced degradation studies confirmed the stability-indicating nature of the method, with both drugs showing maximum degradation under acidic stress conditions. Further the method was evaluated for greenness using Greenness metric tools AGREE and AGREE Prep and found the method is ecofriendly. The proposed method provides comprehensive simultaneous chromatographic profiling of pharmacopeial, process-related, degradation, and mutagenic potential azido process impurities of Sacubitril and Valsartan in a single analytical run. Green analytical analysis also strengthens the method for regular practice. The key strength of the developed RP-HPLC method is its ability to simultaneously separate and quantify Sacubitril, Valsartan, and twelve specified impurities which includes 2 mutagenic impurities, while providing comprehensive impurity profiling, stability-indicating capability, RRF determination, and green analytical assessment in a single validated procedure. For profiling of mutagenic impurities usually special equipment like LC-MS/MS will be chosen, but our method mutagenic impurities were quantified using simple HPLC method along with other degradation impurities.....

Keywords: RP-HPLC, RRF, Valsartan, Sacubitril, LOD, LOQ, AGREE, AGREE Prep, etc.....

How to cite this article: Gowd AVK, Khalivulla SI., A Greenness Evaluated Stability-Indicating RP-HPLC Method for Simultaneous Determination of Related Substances Including Potential Azido Process Impurities in Sacubitril–Valsartan Combination... Int J Drug Deliv Technol. 20yy;16(xxs): 649-655. DOI: 10.25258/ijddt.16.xxs.xx

Source of support: Nil.

Conflict of interest: Nil.

INTRODUCTION

Heart failure (HF) is a progressive cardiovascular disorder associated with significant morbidity, mortality, and healthcare burden worldwide. Pharmacological management of heart failure with reduced ejection fraction (HFrEF) has evolved considerably with the introduction of angiotensin receptor-neprilysin inhibitors (ARNIs), which provide superior clinical outcomes compared with conventional renin-angiotensin system inhibitors. Sacubitril/Valsartan, the first approved ARNI combination, has demonstrated significant reductions in cardiovascular mortality and hospitalization among patients with chronic symptomatic heart failure and has

become a cornerstone in contemporary HF management [1,2].

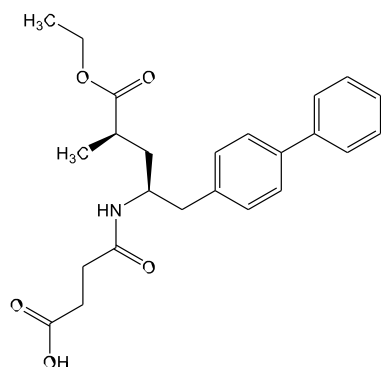


Fig No: 1. Structure of Sacubitril

Sacubitril is a neprilysin inhibitor administered as a prodrug that is rapidly converted to its active metabolite, LBQ657. The active metabolite inhibits neprilysin, thereby preventing the degradation of endogenous vasoactive peptides, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). The resulting enhancement of natriuretic peptide activity promotes vasodilation, natriuresis, diuresis, and suppression of maladaptive neurohormonal activation, collectively reducing cardiac workload and improving cardiovascular function [3]. Chemically, Sacubitril ($C_{24}H_{29}NO_5$; molecular weight 411.5 g/mol) is a white to off-white crystalline powder that is practically insoluble in water but exhibits appreciable solubility in ethanol and dimethyl sulfoxide. Structure was shown in fig No: 1. It possesses a pKa value of approximately 4.6 and a melting point ranging from 166–169°C.

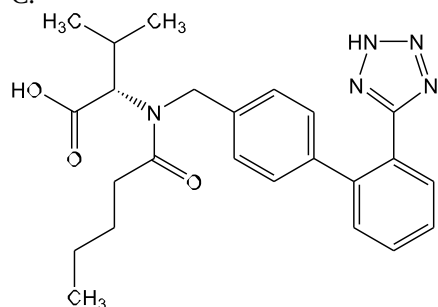


Fig No: 2. Structure of Valsartan

Valsartan is a selective angiotensin II type-1 (AT_1) receptor antagonist widely employed for the treatment of hypertension and heart failure. By selectively blocking the AT_1 receptor, Valsartan inhibits angiotensin II-mediated vasoconstriction, aldosterone secretion, sodium retention, and cardiovascular remodeling, thereby lowering blood pressure and improving cardiac function [4]. Valsartan ($C_{24}H_{29}N_5O_3$; molecular weight 435.5 g/mol) is a white to almost white hygroscopic crystalline powder that is practically insoluble in water and freely soluble in methanol, ethanol, and methylene chloride. The drug exhibits a pKa of approximately 4.73 and a melting point of 116–117°C. Fig No: 2.

The combination of Sacubitril and Valsartan has gained considerable therapeutic importance; however, ensuring its quality, safety, and efficacy requires robust analytical methods capable of detecting process-related impurities,

degradation products, and potential mutagenic impurities at trace levels. According to the International Council for Harmonisation (ICH) guidelines Q3A(R2), Q3B(R2), M7(R2), and Q2(R2), impurities present in pharmaceutical products should be identified, quantified, and controlled using validated stability-indicating analytical procedures to ensure patient safety throughout the product lifecycle [5–8].

Several chromatographic methods have been reported for the simultaneous estimation of Sacubitril and Valsartan in pharmaceutical dosage forms using high-performance liquid chromatography (HPLC) and LC–MS/MS techniques [9–12]. Among these, Prajapati et al. developed an RP-HPLC method employing an Accucore XL C8 column with UV detection at 240 nm for assay determination of Sacubitril and Valsartan tablets [9]. Although these methods provide satisfactory assay determination of the active pharmaceutical ingredients, they primarily focus on drug quantification and do not demonstrate comprehensive separation of process-related impurities, degradation products, or potential mutagenic impurities. Furthermore, the available methods are generally unsuitable for complete impurity profiling required during product development, stability studies, and regulatory submissions.

Analytical method development for fixed-dose combination products presents additional challenges because impurities originating from two chemically distinct active pharmaceutical ingredients must be simultaneously resolved in the presence of formulation excipients. Achieving adequate sensitivity for trace-level impurity quantification often necessitates the preparation of highly concentrated sample solutions. Such high sample loading may introduce excessive placebo components into the chromatographic system, resulting in column contamination, increased back pressure, reduced column lifetime, and compromised analytical performance. Therefore, development of a sensitive, selective, and cost-effective HPLC method capable of quantifying related substances at lower sample concentrations while maintaining complete impurity resolution is highly desirable.

Azido Nitrile Impurity (AZBC) and Azido Tetrazole Impurity (AZBT) are azido process-related impurities associated with the tetrazole synthesis pathway of valsartan. These impurities have attracted significant regulatory attention because of their potential mutagenic risk under the ICH M7 guideline as shown in the figure no: 3. Among them, AZBT has been demonstrated to be Ames-positive and is classified as an ICH M7 Class 2 mutagenic impurity, whereas AZBC is regarded as a potential mutagenic impurity due to its structural alert and common synthetic origin [13]. Therefore, sensitive analytical methods capable of monitoring these impurities are essential for ensuring the quality and safety of sartan drug substances and drug products. To the best of our knowledge, this method is among the few reported RP-HPLC methods capable of simultaneously separating and quantifying Sacubitril, Valsartan, and twelve specified process- and degradation-related impurities (14

chromatographic components) in a single validated, stability-indicating, and green analytical procedure

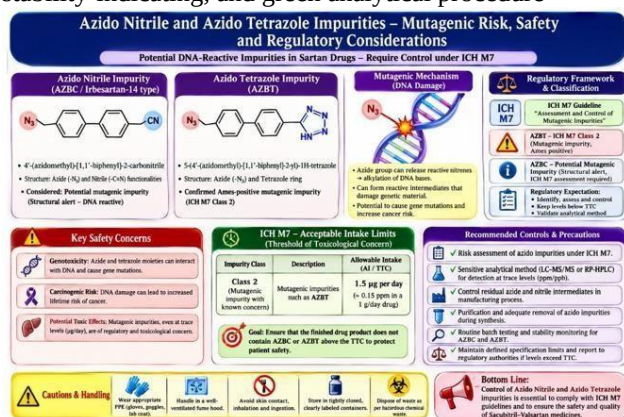


Fig No: 3. Showing Azido nitrile and Azide tetrazole impurities considerations

Although several RP-HPLC methods have been reported for the assay, stability studies, and limited impurity determination of Sacubitril–Valsartan, most are restricted to a limited number of pharmacopeial impurities, stereoisomers, or degradation products. The present study extends the impurity profiling approach by developing a comprehensive stability-indicating RP-HPLC method capable of the simultaneous separation of pharmacopeial impurities, process-related impurities, degradation products, and the potential mutagenic azido impurities,

namely Azido Nitrile Impurity and Azido Tetrazole Impurity, associated with Valsartan, together with the related impurities of Sacubitril, in a single chromatographic run. The proposed method therefore provides a practical and comprehensive analytical platform for routine quality control, stability evaluation, and impurity profiling of Sacubitril–Valsartan pharmaceutical formulations.

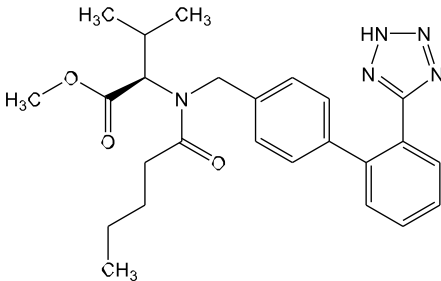
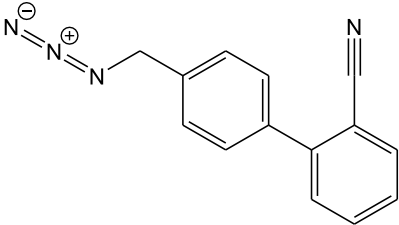
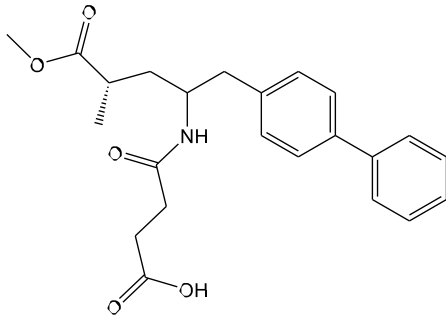
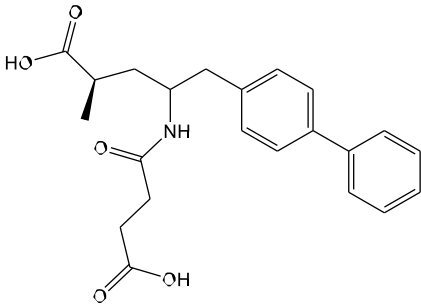
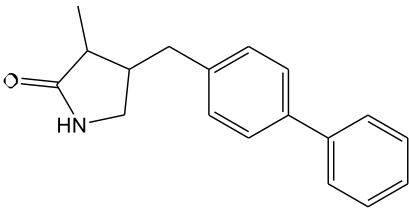
Materials &Method:

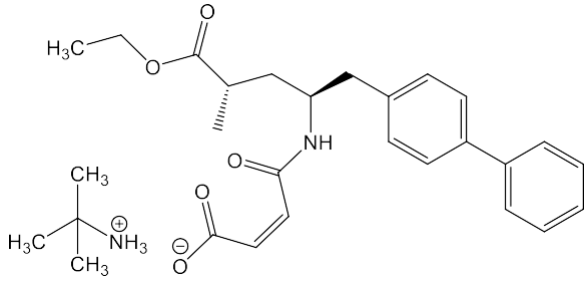
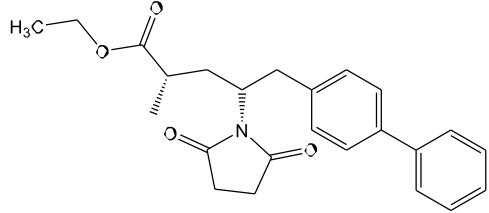
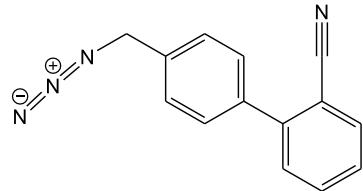
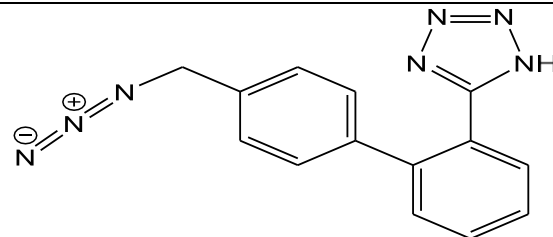
Instrumentation: The chromatographic system consisted of a thermo fischer chromatograph equipped with LC-20AD pumps and an SPD-20A photo diode array (PDA) detector. Samples were injected through a Rheodyne 7725 injector valve via a 100 µL loop. The chromatographic signal was integrated by Chromeleon software. Solubility of the analytes was improved by sonication on an ultrasonicator (Lab man Instruments). Sartorius balance was used to weigh the standards and samples.

Chemicals and reagents: The reference standard samples of Sacubitril and Valsartan were obtained from synzeal. HPLC grade (J.T Baker) Acetonitrile and Methanol were used, while Sodium hydroxide, hydrogen peroxide and Hydrochloric acid were of GR grade (Merck Ltd.). Water (Milli-Q grade) was used throughout the analysis. The impurities details were shown in the Table No: 1.

Table No: 1. Showing the impurities details:

S.No	Name of the impurity	Structure	Source
	Desvaleryl Impurity		Valsartan
	Valsartan Related compound B or EP Impurity C		Valsartan
	Valsartan Related compound C or EP Impurity B or Benzyl Impurity		Valsartan

	Valsartan Methyl ester Impurity or Valsartan USP related compound E		Valsartan
	Irbesartan Impurity 14		Valsartan
	Methyl Impurity (or) Sacubitril Methyl ester		Sacubitril
	Diacid Sacubitril Des ethyl Impurity		Sacubitril
	Cyclic Impurity /Lactam Impurity		Sacubitril

Malic analogue or Maleic Impurity		Sacubitril
Dehydri Impurity or Sacubitril Succinamide Impurity		Sacubitril
Azido nitrile Impurity (Mutagenic)		Valsartan
*Azido Tetrazole Impurity (Mutagenic)		Valsartan

olubility Studies: A Sacubitril and Valsartan mixture was prepared as almost white to crystalline white powder which is freely soluble in water, methanol and practically insoluble in Dichloromethane. Solubility studies were performed at different pH conditions and in different solvents at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. As the figure below shows it is found to be freely soluble in phosphate buffer pH 7.2 and moderately soluble in phosphate buffer pH 8.0 and insoluble on pH buffers 1.2, 3.0 and 4.5. The figure also depicts that the mixture is freely soluble in water, soluble in methanol and practically insoluble in 0.1N HCl and Dichloromethane. The solubility profile was shown in Figure No: 4

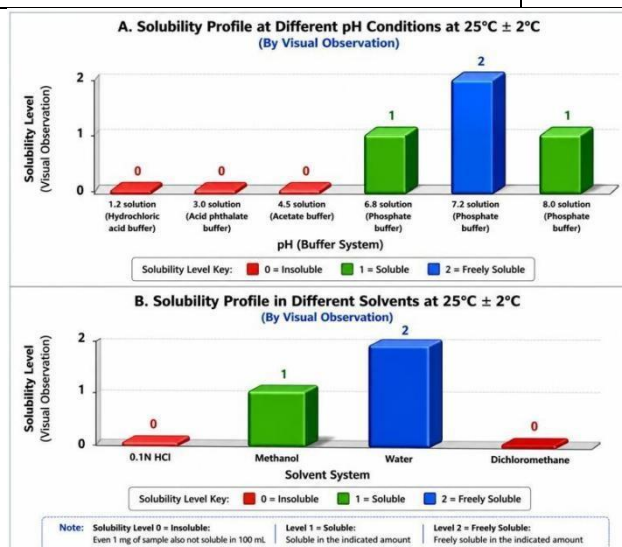


Fig No: 4. Solubility studies of Sacubitril and Valsartan mixture

Preparation of Solutions:

Specification limits for known, unknown impurities Sacubitril and Valsartan at 0.2%, of respective concentrations. At the concentration of sacubitril (960ppm) and Valsartan (1028ppm), all the impurities are visible and can be quantifiable and hence below

sample preparation was finalised. A Sample solution (For 24.3 mg/25.7 mg) was prepared by Weighing tablet powder equivalent to about 48mg of Sacubitril to a 50 mL volumetric flask. Add about 30 mL of diluent and sonicate for 45 min (Maintain sonication temperature between 10°C to 15°C) with intermittent shaking for every 5 min interval. Allow to equilibrate to room temperature and dilute up to the volume with diluent, mix well to form a homogenous mixture. Centrifuge a portion of sample at 4000rpm for 10 minutes and collect the supernatant, inject into HPLC. (Concentration of Sacubitril is 960µg/ mL and Valsartan 1028 µg/mL)

LOQ Values are finalised based on the daily dose of drug substances and ICH Reporting threshold limit. LOQ is 0.05% for both sacubitril and Valsartan impurities and should prove precision and Accuracy for all impurities at the specified concentrations in the Method Validation. On the Basis of above considerations below chromatographic conditions and method of sample preparation was finalised.

Preparation of Mobile Phase:

Mobile phase A: Prepare a mixture of water, methanol and orthophosphoric acid (500 : 500 : 1 v/v/v) and degas by sonication.

Mobile phase B: Prepare a mixture of water, methanol, Acetonitrile and orthophosphoric acid (250: 330: 420: 1 v/v/v/v), and degas by sonication. The Method was optimized as after many trails as shown in Fig No: 5.

Trials	Mobile Phase	Mode of Elution	Reason for rejection																					
1 Trial 1	Mobile Phase: Acetonitrile: Water (40:60 v/v) with Kromasil C8 (150mm x 4.6mm, 5µ) at 225nm	Isocratic 	✗ Impurities are not separated																					
2 Trial 2	Mobile phase: ACN: Water (60:40 v/v) at 225nm	Isocratic 	✗ Peak shape is not good, Peak broadening.																					
3 Trial 3	Mobile phase: ACN: water (70:30 v/v) at 225nm	Isocratic 	✗ Two Analytes peaks were separation with good peak shape, But impurities are not separated																					
4 Trial 4 (Optimized)	Mobile phase A: Water, methanol and OPA (500 : 500 : 1 v/v/v) Mobile phase B: water, methanol, acetonitrile and OPA (250 : 330 : 420 : 1 v/v/v/v) with Kromasil C18 (150mm x 4.6mm, 5µ)	Gradient <table><thead><tr><th>Time (min)</th><th>A (%)</th><th>B (%)</th></tr></thead><tbody><tr><td>0.0</td><td>98.0</td><td>2.0</td></tr><tr><td>18.0</td><td>98.0</td><td>2.0</td></tr><tr><td>45.0</td><td>75.0</td><td>25.0</td></tr><tr><td>75.0</td><td>15.0</td><td>85.0</td></tr><tr><td>76.0</td><td>98.0</td><td>2.0</td></tr><tr><td>85.0</td><td>98.0</td><td>2.0</td></tr></tbody></table>	Time (min)	A (%)	B (%)	0.0	98.0	2.0	18.0	98.0	2.0	45.0	75.0	25.0	75.0	15.0	85.0	76.0	98.0	2.0	85.0	98.0	2.0	✓ All the impurities are injected individually including both the Standards ✓ All are separated with each other.
Time (min)	A (%)	B (%)																						
0.0	98.0	2.0																						
18.0	98.0	2.0																						
45.0	75.0	25.0																						
75.0	15.0	85.0																						
76.0	98.0	2.0																						
85.0	98.0	2.0																						

Column: Kromasil C18 (150 mm x 4.6 mm, 5 µm) | Detection: 225 nm | Mode of Elution: Isocratic (Trials 1–3), Gradient (Trial 4)

Column: Kromasil C18 (150 mm x 4.6 mm, 5 µm) | Detection: 225 nm | Mode of Elution: Isocratic (Trials 1-3), Gradient (Trial 4)

Figure No:5. Showing trails information of Method

Gradient programme: A gradient elution was employed at a constant flow rate of 1.3 mL/min. The mobile phase was initially set at 98% A and 2% B (0–18 min), changed to 75% A and 25% B at 45 min, further adjusted to 15% A and 85% B at 75 min, and then returned to the initial composition (98% A and 2% B) at 76 min. The column was re-equilibrated under the initial conditions until the end of the run at 85 min

Diluent: Water and Acetonitrile (75: 25 v/v).

Valsartan Desvaleryl, Sacubitril Diacid, Sacubitril Cyclic, Sacubitril Succinamide Impurity stock solutions: Individual stock solutions of Valsartan Desvaleryl, Sacubitril Diacid, Sacubitril Cyclic, and Sacubitril Succinamide impurities were prepared by dissolving approximately 2 mg of each impurity in diluent and making up to 20 mL in separate volumetric flasks.

Peak identification solution: Approximately 51.5 mg each of Sacubitril hemicalcium and Valsartan reference

standards were dissolved in diluent and transferred to a 50 mL volumetric flask. One milliliter each of the impurity stock solutions was added, and the volume was adjusted with diluent. Standard and impurity quantities were corrected for potency and salt content.

Standard solution: Stock solutions were prepared by dissolving approximately 40 mg each of Sacubitril hemicalcium and Valsartan reference standards separately in 200 mL of diluent. A diluted standard containing 2 ppm of each analytes was prepared by appropriate serial dilution, corresponding to 0.2% of the test concentration. A sensitivity solution (0.05% of the test concentration) was prepared by further diluting the diluted standard solution.

Placebo preparation: Placebo equivalent to 48 mg of Sacubitril was transferred to a 50 mL volumetric flask, extracted with 30 mL of diluent by sonication for 45 min at 10–15°C, diluted to volume, and centrifuged at 4000 rpm for 10 min. The supernatant was filtered through a 0.45 µm nylon or PVDF syringe filter (3 mL discard) before HPLC injection.

Sample Preparation: Twenty tablets were weighed, powdered, and an amount equivalent to 48 mg of Sacubitril was transferred to a 50 mL volumetric flask. The sample was extracted with 30 mL of diluent by sonication for 45 min at 10–15°C, diluted to volume, mixed thoroughly, and centrifuged at 4000 rpm for 10 min. The supernatant was filtered through 0.45 µm nylon or PVDF syringe filter (3 mL discard) and injected immediately into the HPLC system. Tablet powder was processed within 2 min of crushing to minimize degradation.

Procedure: Inject 10 µL of Diluent, Sensitivity solution, Peak identification solution, diluted standard solution, placebo solutions and Sample solution into the chromatograph and record the chromatograms. Disregard peaks due to diluent and placebo solution. Disregard the impurity peaks below LOQ level for both Sacubitril and Valsartan. Then the % Impurity was calculated from the given formula.

Validation of Proposed Method:

The proposed method was validated for the parameters like System Suitability, Specificity, precision, Accuracy, Linearity, RRF establishment, method precision, intermediate precision, LOD and LOQ. All the validated parameters are found to be in limits according to ICH guidelines.

System Suitability and System Precision: Prepared diluted standard solution of Sacubitril and Valsartan as per test procedure and injected 6 replicate injections. The peak area counts of Sacubitril and Valsartan from six replicate injections of diluted standard solution was calculated. The relative standard deviation of six replicate injections for Sacubitril at the 254nm and Valsartan at 225 nm should not be more than 5.0 %. The data was represented in Table no: 4.

Specificity: Specificity was performed by preparing Blank (diluent), placebo solutions, diluted standard solution, all known impurity solutions individually, sample solution (as such) and sample solution spiked

with all known impurities at specification level and injected into the HPLC equipped with a photodiode array detector and analyzed as per methodology. The retention time of Sacubitril and Valsartan peak obtained in sample solution was compared with Retention times obtained with diluted standard solutions. The retention time of all the known impurities spiked in the sample solution were compared with retention times obtained with individually impurities. Fig No: 6 to 15 depicts the specificity.

Method Precision: Six sample solutions were prepared by spiking all known impurities of Sacubitril and Valsartan at specification level on sample and analyzed as per methodology. % Impurity of each known impurity and % RSD (n=6) for results of six sample solutions was calculated. % RSD (n=6) for % of each known impurity in six sample solutions should be NMT 10. Table No: 5 and 6 depicts method precision.

Intermediate Precision: Six sample solution were prepared by spiking known impurities of Sacubitril and Valsartan at the specification level, from a single lot (same lot used for Method Precision) of Sacubitril and Valsartan Tablets (24.7/25.3 mg) as per methodology by using different column of same make on a different instrument on different day and injected. Data Evaluation % w/w of each known impurity of Sacubitril and Valsartan and % RSD (n=6) for results of six sample solutions was calculated. % RSD (n = 6) for % w/w known impurities of Sacubitril and Valsartan in six spiked sample solutions should be NMT 10.0. Overall % RSD (n = 6 + 6) for % w/w known impurities of Sacubitril and Valsartan in six spiked sample solutions from Method Precision and Intermediate Precision should be NMT 15.0. The detail calculation was in Table No: 7 and 8.

Accuracy: Stock solutions of all known impurities of Sacubitril and Valsartan were prepared and spiked into sample solution to achieve the concentration of impurities in the range of LOQ to 150.0 % for specification. All the accuracy level was prepared in triplicate and single injection was injected.

For all levels individual and mean recovery should be between 85.0 % and 115.0 %. % RSD (n=3) of % recovery at each level should be NMT 10.0. Overall mean recovery (except LOQ) should be between 85.0 % to 115.0%. The Calculation and recoveries were given in the Table No: 9.

Linearity: A series of solutions were prepared by quantitatively diluting the stock solution of Sacubitril and Valsartan working standards, their respective impurities to obtain solutions in the range of about LOQ to 150% specification level of each injected. A Linearity graph was plotted for peak area counts against concentration for each analyte peak. Statistically evaluated the Slope, Correlation coefficient. Method meets the acceptance criteria of linearity for Sacubitril, Valsartan and their impurities. Hence the method is linear for Sacubitril, Valsartan and their impurities over the range. The Linear ranges and data were provided in the Table No: 10. Fig No: 16 depicts overall related compounds linearity graph.

RRF Establishment:

RRF of the impurities were calculated by using slope of the impurity against their respective active component.

Formula for Response factor(RF)

$$= \frac{\text{Slope of Standard from Linearity}}{\text{Slope of Impurity from Linearity}}$$

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

LOQ Values are finalised based on the daily dose of drug substances and ICH Reporting threshold limit. LOQ is 0.05% for Sacubitril and Valsartan impurities. To prove the precision at LOQ and LOD concentration level, Limit of Quantitation (LOQ) was established by injecting six replicates of a solution containing Sacubitril and its known impurities, Valsartan and its known impurities having a concentration at 0.05. Limit of Detection (LOD) was established by quantitatively diluting the LOQ solution and injecting six replicates of a solution containing Sacubitril and Valsartan and its known impurities. The limits for quantification and Detections were depicted in Table No: 11, 12 and 13.

Forced degradation studies: Forced degradation was performed on Sacubitril and Valsartan tablets (24.3 mg/25.7 mg) along with placebo samples subjected to identical stress conditions. The control sample was prepared by randomly selecting 20 tablets, determining the average weight, crushing them into a fine powder, and transferring an amount equivalent to 48 mg of Sacubitril into a 50 mL volumetric flask. The powder was extracted with diluent by sonication for 45 min (maintained at 10–15°C), diluted to volume, centrifuged at 4000 rpm for 10 min, and the supernatant was analyzed by HPLC. Acid degradation was carried out using 1 N HCl at 80°C for 60 min followed by neutralization with 1 N NaOH, alkali degradation using 0.02 N NaOH for 90 min followed by neutralization with 0.02 N HCl, peroxide degradation using 10% H₂O₂ for 90 min, photolytic degradation by exposing the sample to light, thermal degradation by exposing the sample to elevated temperature, and humidity degradation at 93% RH for 48 h. After each stress treatment, samples were diluted, centrifuged, and analyzed using HPLC. Nylon or PVDF syringe filters (0.45 µm) with a 3 mL discard volume were used for filtration. The degradation amounts were mentioned in the Table No: 14 and 15.

Data evaluation and conclusion: Peak purity of Sacubitril, Valsartan, and their known impurities was assessed in all stressed samples, and the percentage degradation was calculated to evaluate the stability-indicating capability of the method. The chromatographic results demonstrated effective separation of degradation products from the analyte peaks with satisfactory peak purity, confirming spectral homogeneity. These findings establish that the proposed HPLC method is specific, stability-indicating, and suitable for the analysis of Sacubitril and Valsartan in the presence of their degradation products under various stress conditions

Greenness Assessment: The Analytical GREENness Metric (AGREE), introduced by Pena-Pereira *et al.* in 2020[14], AGREE integrates all twelve GAC principles

into a single circular pictogram and provides an overall greenness score ranging from 0 (least green) to 1 (most green). Each segment of the circular diagram represents one principle of GAC and is color-coded from red (poor performance) through yellow (moderate performance) to green (excellent performance). The central numerical score reflects the overall greenness of the analytical method. AGREE facilitates the identification of environmentally critical aspects of an analytical procedure, enabling researchers to optimize solvent consumption, minimize hazardous reagents, reduce energy requirements, and improve overall sustainability while maintaining analytical performance. AGREEprep is an extension of the AGREE metric specifically designed to assess the environmental sustainability of sample preparation procedures Developed by Wojnowski *et al.* in 2022 [15], AGREEprep evaluates the greenness of sample

preparation based on the 10 principles of Green Sample Preparation (GSP). Similar to AGREE, the results are displayed as a circular pictogram with an overall greenness score ranging from 0 to 1, where higher values indicate more sustainable sample preparation practices. The evaluation considers factors such as sample size, solvent type and volume, energy consumption, waste generation, process automation, operator safety, and the use of renewable or reusable materials. The scores 0.76 for AGREE and 0.71 for AGREE Prep indicates the method is environment friendly and not causing any hazardous problems to biosystem. The evaluated scores were mentioned in the Figure No: 17 and 18

Results:

Specificity and System Precision:

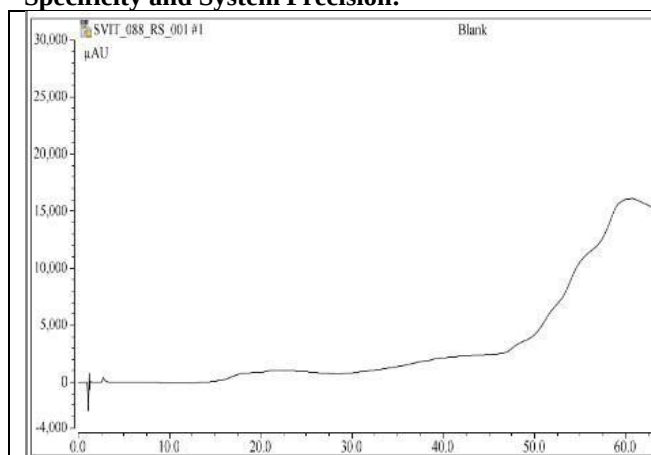


Fig No: 6. Blank Chromatogram at 225nm

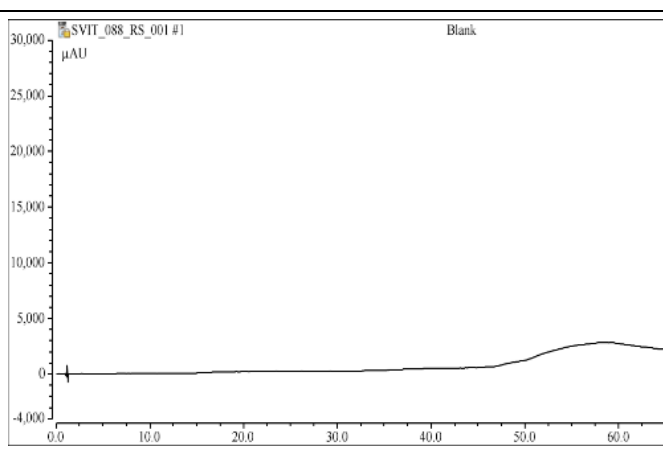


Fig No: 7. Blank Chromatogram at 254nm

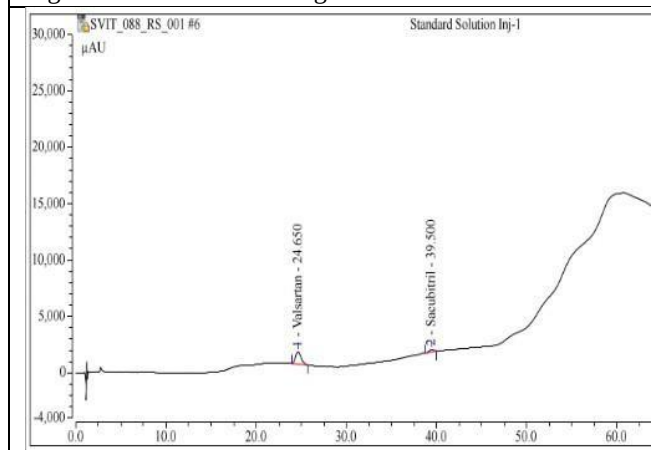


fig No: 8. Standard Chromatogram at 225nm

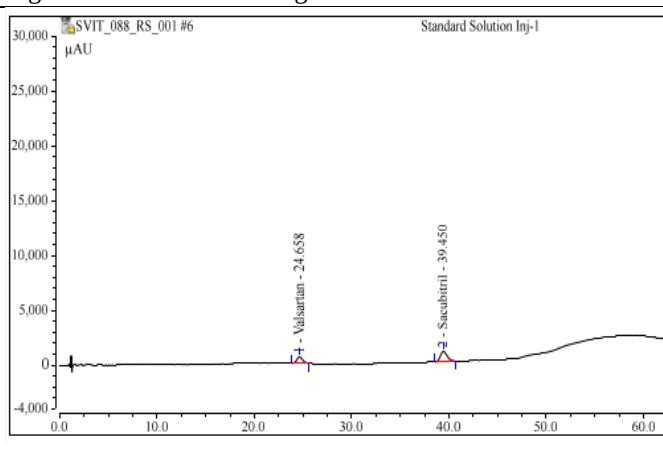


Fig No:9. Standard Chromatogram at 254nm

A Greenness Evaluated Stability-Indicating RP-HPLC Method for Simultaneous Determination of Related Substances Including Potential Azido Process Impurities in Sacubitril–Valsartan Combination....

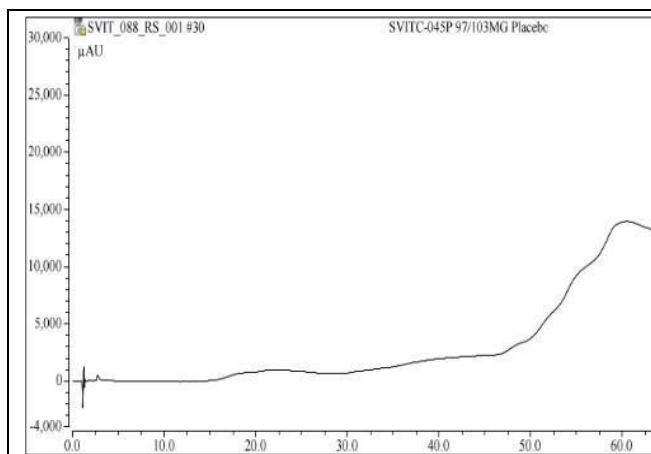


Fig No: 10. Placebo Chromatogram at 225nm

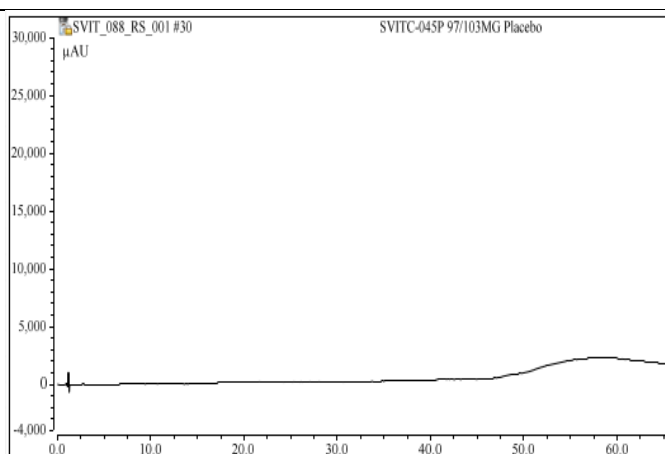


Fig No: 11. Placebo Chromatogram at 254nm

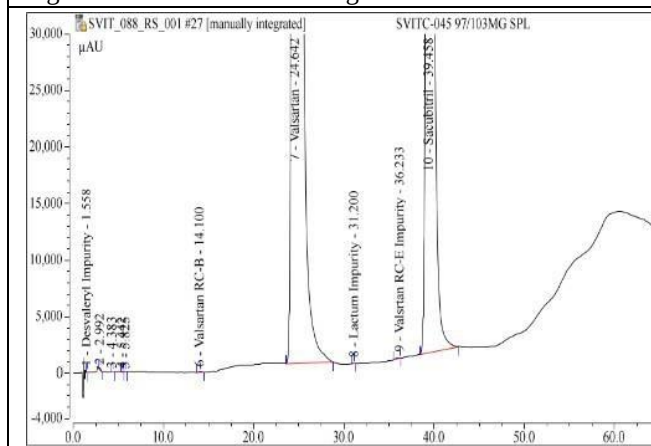


Fig No: 12. Sample Chromatogram at 225nm

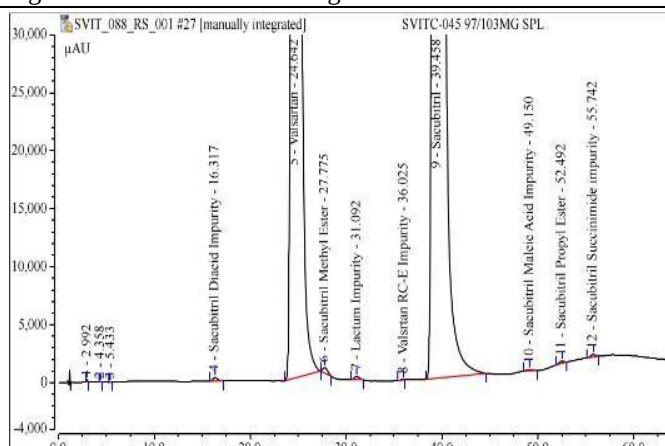


Fig No: 13. Sample Chromatogram at 254nm

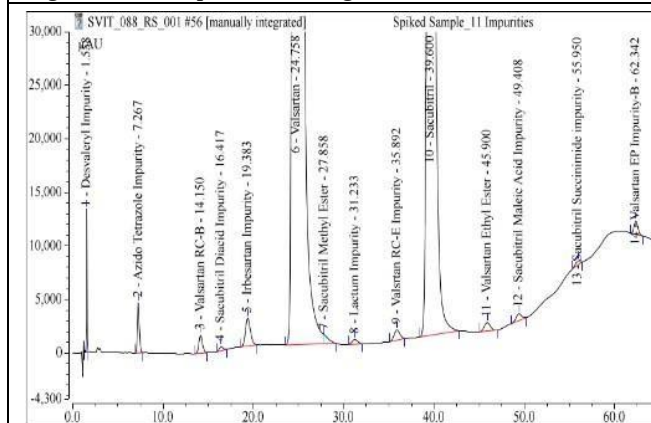


Fig No: 14. Spiked Chromatogram at 225nm

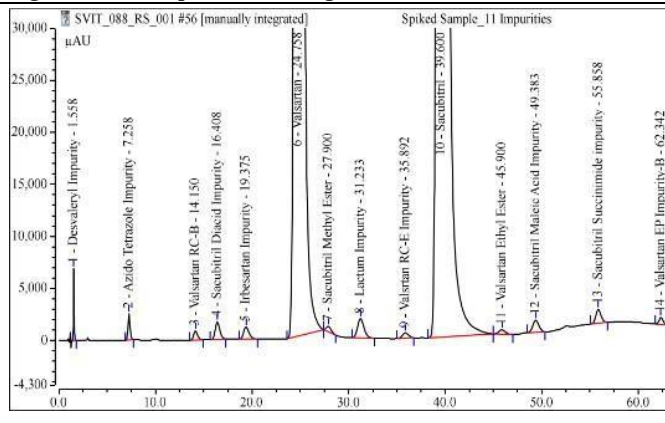


Fig No: 15. Spiked Chromatogram at 254nm

Table No: 2. Retention time Comparison of Sacubitril and Valsartan

Retention time (min)			
Name of compound	Sample solution		Diluted standard solution
	Unspiked	Spiked	
Sacubitril (254 nm)	39.458	39.600	39.450
Valsartan (225 nm)	24.642	24.758	24.650

Table No: 3. Retention time Comparison of Impurities

S. No	Component	Name of the Impurity	Abbreviated form	RT	RRT
1.	Valsartan (225nm)	Desvaleryl Impurity	DSV	1.558	0.06
2.		Valsartan Related compound B or EP Impurity C	RC B	14.150	0.57

3.		Valsartan Related compound C or EP Impurity B or Benzyl Impurity	RC C	62.342	2.52
4.		Valsartan Methyl ester Impurity or Valsartan USP related compound E	RC E	35.892	1.45
5.		Valsartan	VAL	24.758	1.00
6.	Sacubitril (254nm)	Methyl Impurity (or) Sacubitril Methyl ester	SME	27.900	0.70
7.		Diacid Impurity or Sacubitril Des ethyl Impurity	DIA	16.408	0.41
8.		Cyclic Impurity /Lactum Impurity	CYC	31.233	0.79
9.		Malic analogue or Maleic Impurity	MAL	49.383	1.25
10.		Dehydro Impurity or Sacubitril Succinamide Impurity	SUC	55.858	1.41
11.		Azido nitrile Impurity or Irbesartan 14	ANI	19.375	0.49
12.		Azido Tetrazole Impurity	ATI	7.258	0.18
13.		Sacubitril	SAC	39.600	1.00

No interference was found at the retention time of analytes and all the impurities were separated from analyte and each other.

Table No:4. Showing System Precision

S. No	Sacubitril area (254 nm)	Valsartan area (225 nm)
1	43605	47872
2	42140	48512
3	43651	48514
4	42178	45214
5	43201	49854
6	41214	48514
Mean	42665	48080
%RSD	2.29	3.22

% RSD (n=6) for peak area counts of Sacubitril and Valsartan from six replicate injections of diluted standard solution was found within acceptance criteria for System Precision, Hence the method is precise.

Method Precision:

Table No:5. Showing Method precision of Valsartan and related compounds

Valsartan				
Nominal Concentration	0.2 %w/w	0.2 %w/w	0.2 %w/w	0.2 %w/w
	DSV	RC B	RC C	RC E
Mean (n=6)	0.208	0.206	0.204	0.206
SD	0.01	0.01	0.01	0.01
RSD	4.9	4.8	3.5	4.7

Table No:6. Showing Method precision of Sacubitril and related compounds

Sacubitril							
Nominal Concentration	0.2% w/w	0.2 %w/w	0.2% w/w	0.2% w/w	0.2% w/w	0.2% w/w	0.2% w/w
	SME	DIA	CYC	MAL	SUC	ANI	ATI
Mean (n=6)	0.204	0.209	0.205	0.207	0.204	0.204	0.203
SD	0.01	0.01	0.01	0.01	0.01	0.01	0.01
RSD	4.9	3.7	3.8	4.3	4.0	3.9	2.9

%RSD (n=6) for % of each known impurity in six sample solutions was found within acceptance criteria.

Intermediate Precision:

Table No:7. Showing Intermediate precision of Valsartan and related compounds

Valsartan				
Nominal Concentration	0.2 %w/w	0.2 %w/w	0.2 %w/w	0.2 %w/w
	DSV	RC B	RC C	RC E
Mean (n=6)	0.205	0.200	0.202	0.203
SD	0.01	0.01	0.01	0.01
RSD	4.5	3.4	4.5	3.4

Table No:8. Showing method precision of Sacubitril and related compounds

Sacubitril							
Nominal Concentration	0.2% w/w	0.2 %w/w	0.2% w/w	0.2% w/w	0.2% w/w	0.2% w/w	0.2% w/w
	SME	DIA	CYC	MAL	SUC	ANI	ATI
Mean (n=6)	0.203	0.204	0.205	0.202	0.202	0.205	0.203
SD	0.01	0.01	0.01	0.01	0.01	0.01	0.01
RSD	4.8	4.0	3.5	3.0	3.5	4.9	2.9

Accuracy:

Table No:9. Showing Recovery data of Valsartan and Sacubitril related compounds

Impurity	Level	LOQ	50%	100%	150%
DSV	% Mean recovery	100.54	99.88	98.78	97.9
	%RSD	1.065	2.0162	0.961	0.791
RCB	% Mean recovery	101.46	100.55	99.43	98.04

	%RSD	1.44	1.494	0.826	0.685
RCC	% Mean recovery	100.34	100.13	100.81	98.32
	%RSD	0.999	100.137	1.855	0.738
RCE	% Mean recovery	97.89	99.62	98.57	98.34
	%RSD	0.973	0.771	1.142	0.801
VAL	% Mean recovery	100.32	99.4	100.66	99.41
	%RSD	1.222	0.854	0.696	1.42
SME	% Mean recovery	101.15	100.63	99.26	98.08
	%RSD	1.108	2.085	1.194	1.003
DIA	% Mean recovery	101.08	100.86	101.42	99.17
	%RSD	1.054	2.031	1.233	1.017
CYC	% Mean recovery	99.33	100.36	101.55	98.1
	%RSD	0.929	1.491	1.172	0.96
MAL	% Mean recovery	100.45	100.13	98.73	97.19
	%RSD	2.43	1.172	1.676	1.231
SUC	% Mean recovery	99.98	100.18	101.25	98.6
	%RSD	0.393	0.921	1.39	1.40
ANI	% Mean recovery	99.98	101.98	101.13	99.7
	%RSD	0.459	4.642	1.449	2.304
ATI	% Mean recovery	100.05	100.1	99.91	99.92
	%RSD	1.306	0.834	0.57	2.424
SAC	% Mean recovery	101.73	100.63	98.98	98.4
	%RSD	0.841	2.085	0.717	0.41

For all levels individual and mean recovery was found to be within acceptance criteria. % RSD (n=3) of % recovery at each level was found to be within acceptance criteria. Overall mean recovery found to be between 85.0 % to 115.0%.

Linearity:

Table No:10. Showing Linearity of Valsartan and Sacubitril related compounds.

SNO:	Impurity	Linearity Conc. Range (µg/mL)	Slope	Intercept	Correlation Coefficient	RRF
1	DSV	0.50108 - 3.00648	26986.2233	418.3784	1.0000	1.12
2	RCB	0.50228 - 3.01368	23636.3803	128.5405	1.0000	0.98
3	RCC	0.50060 - 3.00360	18575.3042	30.6486	0.9993	0.77
4	RCE	0.50148 - 3.00888	21342.3941	501.8919	0.9993	0.89
5	VAL	0.50470 - 3.02820	24019.7816	560.1081	0.9998	1.00
6	SME	0.50036 - 3.00216	9087.8081	48.8378	1.0000	0.42
7	DIA	0.50076 - 3.00456	27687.9144	336.0000	0.9999	1.27
8	CYC	0.50172 - 3.01032	43231.0420	320.1892	1.0000	1.98
9	MAL	0.50104 - 3.00624	25755.5636	165.7838	1.0000	1.18
10	SUC	0.50028 - 3.00168	25070.3390	114.5946	1.0000	1.15
11	ANI	0.50084 - 3.00504	22770.3134	360.8919	0.9996	1.04

12	ATI	0.50076 - 3.00456	18139.7249	472.3243	0.9996	0.83
13	SAC	0.49920 - 2.99490	21879.6851	16.1351	0.9998	1.00

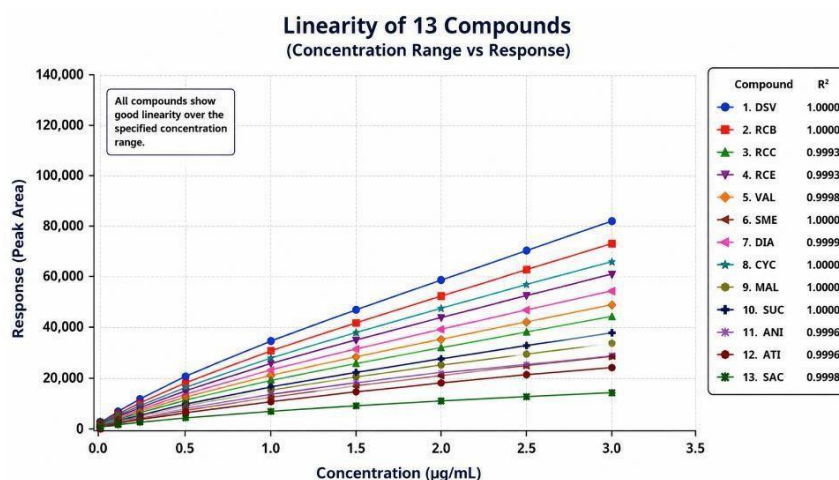


Fig No: 16. Showing the overall Linearity data of all Valsartan and Sacubitril related compounds.

Method meets the acceptance criteria of linearity for Sacubitril, Valsartan and their impurities. Hence the method is linear for Sacubitril, Valsartan and their impurities over the range. RRF of the impurities were calculated by using slope of the impurity against their respective active component.

LOQ and LOQ:

Table No: 11. Showing Limit of Quantification of Valsartan related compounds

	Valsartan									
Nominal Concentration	0.05 %w/w		0.05 %w/w		0.05 %w/w		0.05 %w/w		0.05 %w/w	
	DSV		RC B		RC C		RC E		VAL	
	Area	S/N	Area	S/N	Area	S/N	Area	S/N	Area	S/N
Mean (n=6)	13062.17	12	11918.83	13	9605	11	10599.17	12	11253.83	11
SD	238.2		347.2		356.9		471.5		344.1	
RSD	1.8		2.9		3.7		4.4		3.1	

Table No: 12. Showing Limit of Quantification of Sacubitril impurities

Sacubitril						
SNO:	Nominal Concentration		S/N	Mean area	SD	%RSD
1	0.05 %w/w	SME	10	4548.33	263.1	5.8
2	0.05 %w/w	DIA	15	13480.50	525.7	3.9
3	0.05 %w/w	CYC	40	22719.00	454.5	2.0
4	0.05 %w/w	MAL	15	13088.33	171.0	1.3
5	0.05 %w/w	SUC	15	12080.50	172.2	1.4
6	0.05 %w/w	ANI	13	11562.17	363.5	3.1
7	0.05 %w/w	ATI	10	9208.17	148.4	1.6
8	0.05 %w/w	SAC	15	10725.50	409.3	3.8

Table No: 13. Showing Limit of Detection of Valsartan and Sacubitril impurities

S. No	Component	Name of the Impurity	LOD %w/w Nominal Concentration	Area	S/N
1	Valsartan (225nm)	DSV	0.021	4100	7
2		RC B	0.024	3400	3
3		RC C	0.022	3076	3
4		RC E	0.024	3298	3
5		VAL	0.023	3707	4
6	Sacubitril (254nm)	SME	0.022	1879	3
7		DIA	0.022	4309	7
8		CYC	0.023	7654	9
9		MAL	0.022	4563	7
10		SUC	0.022	4328	7
11		ANI	0.022	3987	4
12		ATI	0.023	3009	3
13		SAC	0.024	3543	3

Table No: 14. Showing forced degradation studies of Sacubitril

Sr. No.	Condition		%Assay of Sacubitril	% Degradation with respect to untreated sample
1	Control Sample		99.01	0.48
2	Acid degradation	1N HCl for 60 minutes at 80°C	87.55	12.179
3	Alkali degradation	0.02 N NaOH; On benchtop for 90 minutes	99.21	0.452
4	Peroxide degradation	10% H ₂ O ₂ On benchtop for 90 minutes	98.52	1.16
5	Photolytic degradation	Exposed the sample to achieve 1.2 million lux.	99.13	0.201
6	Thermal degradation	Exposed the sample at 80°C for 72 Hrs.	99.05	0.681

7	Humidity degradation	Exposed the sample at 93 % RH/25degrees for 48 hours.	99.72	0.198
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Table No: 15. Showing forced degradation studies of Valsartan

Sr. No.	Condition		%Assay of Valsartan	% Degradation with respect to untreated sample
1	Control Sample		100.00	0.028
2	Acid degradation	1N HCl for 60 minutes at 80°C	98.47	1.121
3	Alkali degradation	0.02 N NaOH; On benchtop for 90 minutes	99.33	0.293
4	Peroxide degradation	10% H2O2 On benchtop for 90 minutes	99.66	0.297
5	Photolytic degradation	Exposed the sample to achieve 1.2 million lux.	99.21	0.421
6	Thermal degradation	Exposed the sample at 80°C for 72 Hrs.	98.95	0.811
7	Humidity degradation	Exposed the sample at 93 % RH/25degrees for 48 hours.	99.81	0.124

Greenness Assessment of the method:



Fig No: 17. Showing AGREE technique

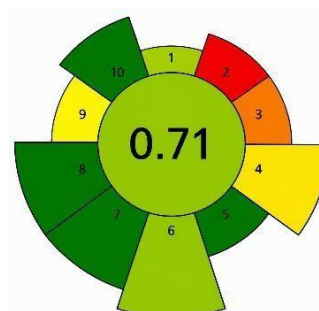


Fig No: 18. Showing AGREE Prep technique

Discussion:

The chromatographic analysis was performed using a Kromasil 100-5 C18 column (150 mm × 4.6 mm, 5 µm; Part No. M05CLA15). The column temperature was maintained at 50°C, while the sample temperature was

kept at 5°C. The mobile phase was delivered at a flow rate of 1.3 mL/min, and detection was carried out using a photodiode array (PDA) detector at 254 nm for Sacubitril and 225 nm for Valsartan. A 10 µL injection volume was employed for each analysis. Separation was achieved

under gradient elution conditions with a total run time of 85 min. The approximate retention times were 25 min for Valsartan and 40 min for Sacubitril. The needle wash solution consisted of water: methanol: acetonitrile (10:45:45, v/v/v), while the column wash solution comprised water: acetonitrile (50:50, v/v) to ensure proper system cleanliness and reproducible chromatographic performance. The developed method was validated according to ICH guidelines. This method effectively separated 13 compounds of which two are mutagenic impurities. % RSD (n=6) for peak area counts of Sacubitril and Valsartan from six replicate injections of diluted standard solution was found within acceptance criteria for System Precision, Hence the method is precise. No interference was found at the retention time of analytes and all the impurities were separated from analyte and each other. %RSD (n=6) for % of each known impurity in six sample solutions was found within acceptance criteria. For all levels individual and mean recovery was found to be within acceptance criteria. % RSD (n=3) of % recovery at each level was found to be within acceptance criteria. Overall mean recovery found to be between 85.0 % to 115.0%. Method meets the acceptance criteria of linearity for Sacubitril, Valsartan and their impurities. Hence the method is linear for Sacubitril, Valsartan and their impurities over the range. RRF of the impurities were calculated by using slope of the impurity against their respective active component. LOQ and LOD are calculated as %W/W as mentioned in the table: X. Forced degradation studies were carried out and found both Sacubitril and Valsartan has more acidic degradation. Furthermore, the method employs an optimized low sample concentration strategy to minimize placebo interference, reduce column contamination, and improve method robustness while maintaining adequate sensitivity for impurity determination. The method was also analysed by green analytical tools like AGREE and AGREE Prep and found the method is ecofriendly.

Conclusion:

The developed gradient RP-HPLC method provides a reliable, specific, accurate, precise, and stability-indicating approach for the simultaneous determination of Sacubitril, Valsartan, and their related impurities in pharmaceutical dosage forms. The method offers excellent chromatographic resolution with satisfactory sensitivity, linearity, accuracy, and robustness, enabling the effective separation of analytes from their impurities and degradation products under various stress conditions. Its validated performance in accordance with ICH guidelines demonstrates its suitability for routine quality control, impurity profiling, release testing, and stability studies. Furthermore, the established Relative Response Factors (RRFs), LOD, and LOQ values enhance its applicability for regulatory compliance and impurity quantification. Owing to its robustness and comprehensive validation, the proposed method can serve as a valuable analytical tool in pharmaceutical research and development, process optimization, formulation development, quality assurance, stability testing, technology transfer, and commercial

manufacturing of Sacubitril and Valsartan formulations, while also providing a strong platform for future adaptation to other combination products and related pharmaceutical analyses

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