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DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHOD FOR ESTIMATION OF DRUGS

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Abstract
A simple, rapid, cost effective and optimized reversed phase liquid Chromatographic (RP-HPLC) method for estimation of Sacubitril and Valsartan is developed. The Chromatographic method development was done on C18 column using Acetonitrile and Water as mobile phase. For method optimization Quality by Design approach was used. The mobile phase ratio, flow rate and pH were identified as critical method parameters (CMPs) and they were optimized for critical analytical attributes which are retention time and peak area using Box Behnken quadratic design. Experimental trials were conducted and response surface analysis was done using design expert version 13 software. The experimental result data were in the range of predicted data. The optimized chromatographic conditions are Acetonitrile: Water (pH 4.5 adjusted with formic acid) 75:25 (v/v) as mobile phase, flow rate of 1 ml/min at 247 nm UV detection with run time of 6 minutes. The method was validated for linearity, accuracy, precision and specificity. The method was found to be linear with the concentration range of 2-10 ppm with a correlation coefficient of 0.998 for both drugs. The overall average accuracy for sacubitril and valsartan was found to be 103.7% and 107.2% respectively which is within the acceptance criteria. The % content of sacubitril and valsartan was found to be 99.05% and 100.7% w/w respectively. The studies include successful use of Quality by Design approach for method optimization and validated method was robust, reproducible and specific to the retention time of drugs.

Keywords: RP-HPLC, Sacubitril, Valsartan, Quality by Design, QbD, Design Expert.

Introduction:

Heart failure is a complex clinical syndrome that results from structural or functional impairment of ventricular filling or ejection. It is a major public health problem associated with a high mortality rate, frequent hospitalizations and poor quality of life. Approximately 30% of all deaths are due to heart failure, with reduced ejection fraction probably the most important modifiable risk factor. A fixed dose combination of sacubitril and valsartan has been approved by FDA for the management of heart failure. It consists of the neprilysin inhibitor sacubitril and the angiotensin receptor blocker valsartan. It is recommended for use as a replacement for an ACE inhibitor or an angiotensin receptor blocker in people with heart failure with reduced ejection fraction. [1]

There are some methods that have been reported for analysis of these drugs in single and combination formulation. Several chromatographic method have been reported for separation and quantification of sacubitril and valsartan,[2-4] its degradation products and related impurities in bulk drugs[5-7] and

biological samples such as animal and human plasma.[8,9] Various liquid chromatography methods reported in literature include HPLC method coupled with UV detector, fluorescence detector,[10] diode array detector,[11] UPLC method,[12] LC-MS-MS method.[13,14] All these methods have disadvantages such as high cost of process and complex instrumentation which takes more time.

Quality by Design is defined as systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management. Quality by Design (QbD) is well established in the pharmaceutical industry for manufacturing processes (ICH Q8 for pharmaceutical development and ICH Q11 for development and manufacture of drug substances). These QbD principles have been applied to the development of analytical methods, and are termed as Analytical QbD. In past few years several method were reported in the literature which contains successful application of Analytical Quality by design approach for development of efficient and cost-efficient liquid chromatography methods for estimation bulk drugs, pharmaceutical formulations, and bioanalytical samples. [15-20] Therefore in the current study Quality by design principles have been applied for the development of high performance liquid chromatography (HPLC) method for Valsartan and Sacubitril. Developed method was validated as per ICH guidelines.

Materials and methods

Drugs, Chemical and Solvents:

The drug samples of Valsartan and Sacubitril were purchased from Yarrow Chem Products, Ghatkopar (W), Mumbai 400086.

The reagents: Acetonitrile (HPLC grade), Methanol (HPLC grade), Water (HPLC grade), Formic acid (HPLC grade) were ordered from Loba Chemie PVT. LTD. Colaba, Mumbai, 400005.

Instruments:

General:

Electronic weighing balance, micropipettes, ultrasonicator, pH meter, were used.

Apparatus:

Beakers, measuring cylinders, glass rod, pipettes, volumetric flasks

UV-Visible Spectrophotometer

A SICAN-2301 spectrophotometer was used for measurement of UV absorbance in the range of 200 to 400nm.

HPLC System

JASCO HPLC-400 with chromnav software was used. The Column used was HiQsil C18 having dimension 4.6mmID x 250mmL 5µm.

Preparation of Standard Stock solution and Standard dilution of Valsartan and Sacubitril

Standard stock solution:

To prepare standard stock solution, 10mg of Valsartan API was weighed and transferred into a 10ml volumetric flask. Mobile phase was prepared having composition acetonitrile: water 75:25 (v/v) (pH 4.5 adjusted with formic acid). About 4ml of mobile phase was added initially to dissolve the drug. Further mobile phase was added to make up the volume up to 10ml.

For Sacubitril 5mg was weighed and transferred into a 5ml volumetric flask. About 2ml of mobile phase was added to dissolve the drug. Further mobile phase was added to make up the volume up to 5ml. Further dilutions were made to get 10 ppm concentration of Valsartan and Sacubitril respectively.

Determination of wavelength maxima of sacubitril and valsartan:

The API of Valsartan and Sacubitril were scanned in the range of 200-400 nm wavelength using methanol as a solvent. Sacubitril showed characteristic absorption maxima at 230 nm and Valsartan showed absorption at 228 nm. When both drugs analysed in combination showed absorption at 247 nm. Hence the wavelength of analysis for Sacubitril and Valsartan was selected as 247 nm.

Selection of Mobile Phase:
Different solvents were used for mobile phase and different strengths were tried for chromatographic analysis of both drugs. Various mobile phase compositions tried for simultaneous estimation of sacubitril and valsartan. Mobile phase compositions used were Methanol: Water (75:30), Methanol: Water (85:15), Acetonitrile: 0.1% formic acid in water (75:25) and Acetonitrile: Water (pH 4.5 adjusted with formic acid) (75:25). From above Mobile phase compositions Acetonitrile: Water (pH 4.5 adjusted with formic acid) (75:25) was used for further analysis.

Chromatographic Conditions:
A HiQsil C18 4.6mmID x 250mmL 5µm column was used as the analytical column. The mobile phase having composition acetonitrile: water (75:25) (v/v) (pH 4.5), was filtered and sonicated prior to use. The flow rate was maintained at 1mL/min and injection volume was 20 µl. The detection was carried out at a 247nm UV detector.

Method Optimization:

Experimental Design:
Design Expert software version 13 was used for method optimization and experimental design was constructed. Mobile phase ratio, pH and flow rate were considered as Critical Method Parameters (CMP) and retention time and peak area responses were considered as Critical Analytical Attributes (CAA). Above CAA and CMPs were considered as input variables in software. Experimental design was constructed to study these factors and to verify method performances. Statistical analysis was done using the Box-Behnken design.
A factorial design consists of three factors, 3 levels were considered for the experimental plan. The levels of three factors are given in Table no. 1.

TABLE 1: EXPERIMENTAL DESIGN PLAN

Factors	Level 1	Level 2	Level 3
Mobile phase (%)	20	25	30
pH	4	4.5	5
Flow rate (mL/min)	0.9	1	1.1

Details of three factor three level factorial design used for experimental plan in method optimization.

Response surface Box-Behnken quadratic design was used to optimize and evaluate the effects of CMPs. The different method conditions (17 runs) obtained from experimental design along with the design plan are shown in Table no. 2. Desired goals for CAAs that are retention time and peak area were set and given to the software.

TABLE 2: BOX-BEHNKEN DESIGN PLAN AND RESPONSES

Std	Run	Factor 1 Mobile Phase ratio (%)	Factor 2 pH	Factor 3 Flow rate (mL/min)	Response 1 Retention time (min)	Response 2 Retention time (min)	Response 3 Peak area	Response 4 Peak area
9	1	25	4	0.9	3.8	4	506859	146421
5	2	20	4.5	0.9	4	4.3	674023	85222
17	3	25	4.5	1	3.7	3.9	603720	40687
4	4	30	5	1	2.5	4	279644	508507
10	5	25	5	0.9	2.7	4	204599	610051
1	6	20	4	1	2.2	3.1	388537	569056
8	7	30	4.5	1.1	1.9	2.6	362342	523877

11	8	25	4	1.1	2	2.8	339165	526461
6	9	30	4.5	0.9	2.9	4.8	379328	688771
13	10	25	4.5	1	2.6	3.9	196075	593617
14	11	25	4.5	1	2.6	3.8	172729	518150
3	12	20	5	1	2.3	3.3	367248	550288
2	13	30	4	1	2.3	3.2	390516	543305
12	14	25	5	1.1	2.2	3.2	341060	496291
7	15	20	4.5	1.1	2	2.9	325445	487861
16	16	25	4.5	1	2.2	3.5	357452	539660
15	17	25	4.5	1	2.5	3.7	390297	548393

The different method conditions (17 runs along with the results) obtained from experimental design used in method optimization.

Method Validation:

Analytical method validation was carried out as per ICH guidelines. The parameters include:

1. System Suitability:

Following factors were selected as system suitability parameters

1. Theoretical plates
2. Tailing factor
3. Resolution

2. Specificity:

It was determined with the help of LLOQ (lower limit of quantification). LLOQ which is 0.5ppm was injected and the blank run was done. Results of both the runs were compared.

3. Linearity:

A calibration curve was constructed using three replicates (n=3) analysis of six standard solutions of 0.5, 2,4,6,8 and 10µg/ml concentration. Peak area versus concentration graph was plotted after which Least squares linear regression analysis of data was undertaken to establish the equation for the best fit line and the correlation coefficient (R^2) was used to confirm linearity.

4. Precision:

Intra-day (repeatability) precision was established following analysis of replicate samples (n=6) at three concentrations indicative of low, medium and high levels within the linear range viz., 3, 5, 8 µg/ml respectively. Analysis was performed over a short period of time on the same day. Inter-day precision or reproducibility was assessed at low, medium and high concentration on three consecutive days and the percent relative standard deviation (% RSD) was used to assess intra and inter-day precision. An upper limit of 2 % RSD was used to confirm precision in our laboratory.

5. Accuracy by recovery:

The accuracy was determined using three different levels (50%, 100%, and 150%) in which formulation concentration was kept constant and amount of API was added in formulation in various amount corresponding to 50%,100% and 150%. 6ppm, 8ppm and 10ppm concentrations were analysed in triplicates and % Accuracy was calculated by recovery method.

6. Limit of Detection and Limit of Quantitation:

LOD and LOQ was determined using calculations based on standard deviation of a response and the slope of the calibration curve. The LOD and LOQ were calculated using 3σ/S and 10σ/S respectively.

7. Stock Solution Stability:

Stock solution stability were determined by keeping it in the refrigerator. The stock solution prepared for analysis was kept in the refrigerator and temperature was maintained at 2^0C to 8^0C. On the next day solution was analysed.

8. Robustness:
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was analysed under different conditions by changing the flow rate, mobile phase composition and pH. The results are presented as %RSD.

Assay:
Tablet of brand name Azmarda (Cipla PVT. LTD.) having label claim of 103mg of valsartan and 97mg of sacubitril was used for assay. Five tablets were weighed accurately and finely powdered. Tablet powder equivalent to 25mg of valsartan and 23.54mg of sacubitril was weighed accurately and taken into a 25ml volumetric flask, acetonitrile as diluent was added and mixture was sonicated to dissolve. Further acetonitrile was added to make up the volume up to 25ml. This solution was considered as a stock solution. From this solution 1ml is taken and diluted up to 10ml with mobile phase. Mobile phase is ACN: water (75:25 v/v) (pH 4.5 adjusted with formic acid). Further 1ml was taken from the above solution and diluted up to 10ml with mobile phase, which was used as a sample solution.
Content of Valsartan and Sacubitril was determined using linear regression method.

Results and discussion:
Method Optimization:
Quality by Design Implementation:
The use of a QbD is to ensure flexibility following method development, so as to facilitate continuous improvement of a product whilst avoiding the need for costly post-approval changes following market authorization. [21]
The present study was done for development of RP-HPLC method for estimation of sacubitril and valsartan. The primary requirement of selection of mobile phase composition is that the drug must be soluble in the mobile phase and the solvents must be compatible with the HPLC system. Mobile phase has a characteristic impact on chromatographic method. Mobile phase composition **[**ACN: Water 75:25 v/v (pH 4.5 adjusted with folic acid)] showed good resolution and hence the method was further optimized using Design expert version 13 software. In order to obtain an ideal chromatographic method CAAs were identified such as retention time and peak area. CMPs were identified according to their influence on CAAs such as mobile phase ratio, pH, and flow rate. Seventeen analytical trials were carried out using CAAs and CMPs parameters. These Experimental runs were completed and the results in terms of responses like retention time and peak area were entered in the software for next statistical evaluation as shown in Table no.2 The results are given in terms of contour plot and the optimized conditions of the chromatographic method are given in the Table no.3. Above results showed that if we use the chromatographic method obtained from the optimized condition then the results of the method are in the predicted range. Retention time was found to be 3.7 min and 4.5 min for valsartan and sacubitril respectively. Peak area was found to be 246738 and 335568 for valsartan and sacubitril respectively.

TABLE 3: OPTIMIZED CHROMATOGRAPHIC CONDITION

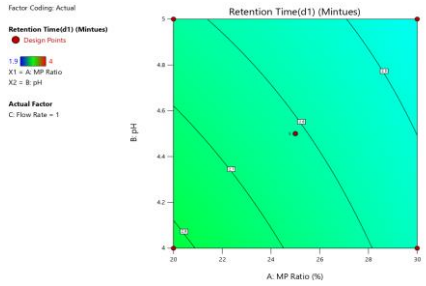
Name	**Solution**
Mobile Phase Ratio	75:25 (v/v)
pH	4.5
Flow Rate	1 ml/min
Retention Time (valsartan)	1.9 to 4 min
Retention Time (sacubitril)	2.6 to 4.8 min
Peak Area (valsartan)	172729 to 674023

Peak Area (sacubitril)	40687 to 688771

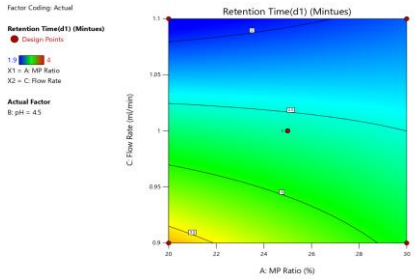
Optimized method conditions after completing the 17 analytical trial runs given by the experimental design.

Contour plots were constructed for each CAAs that is retention time and peak area (fig.1 and fig.2) to understand the interaction of the CMPs and their effect on CAAs and to obtain an optimized chromatographic method.

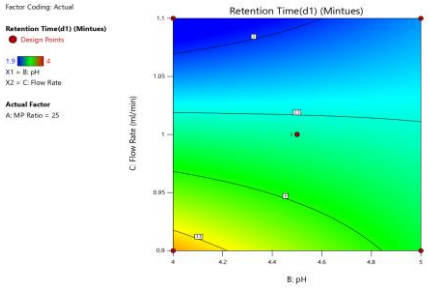
The contour plots of retention time [fig. 1 (1A to 1C valsartan and 2A to 2C sacubitril)] of both drugs depict that if we use flow rate 1ml/min, Mobile phase ratio 75:25v/v, and pH 4.5 then we will get retention time within the set range.



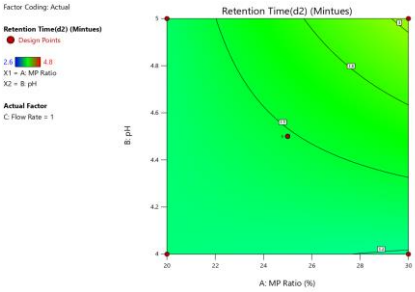
1A: Contour plot of retention time with Flow Rate as actual factor for valsartan



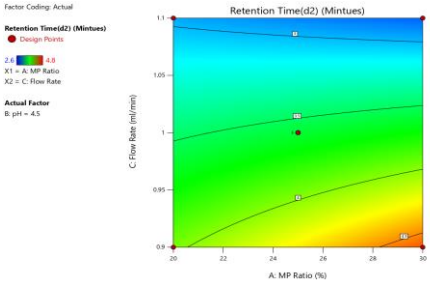
1B: Contour plot of retention time pH as actual factor for valsartan



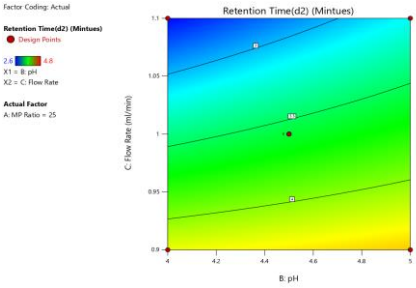
1C: Contour plot of retention time with MP Ratio as actual factor for valsartan



2A: Contour plot of retention time with Flow Rate as actual factor for sacubitril



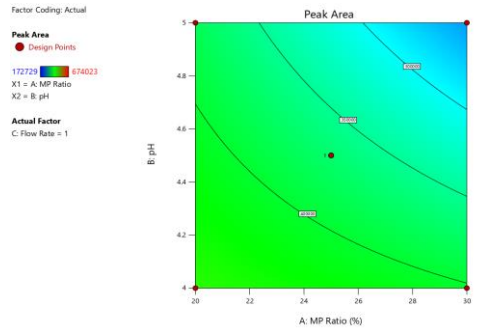
2B: Contour plot of retention time with pH as actual factor for sacubitril



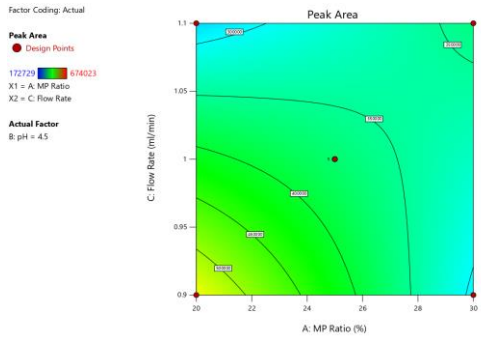
2C: Contour plot of retention time with MP Ratio as actual factor for sacubitril

Figure 1: Contour plots of valsartan and sacubitril for retention time

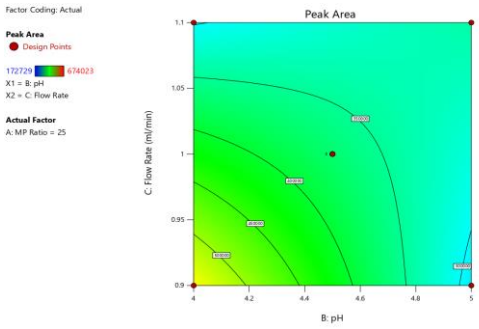
Similarly contour plots of peak area [fig 2 (3A to 3C valsartan and 4A to 4C sacubitril] of both drugs depict that if we take flow rate 1ml/min, Mobile phase ratio 75:25v/v, and pH 4.5 then we will get peak area within the set range.



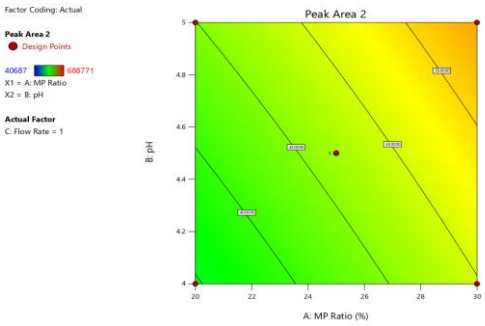
3A: Contour plot of peak area with Flow Rate as actual factor for valsartan



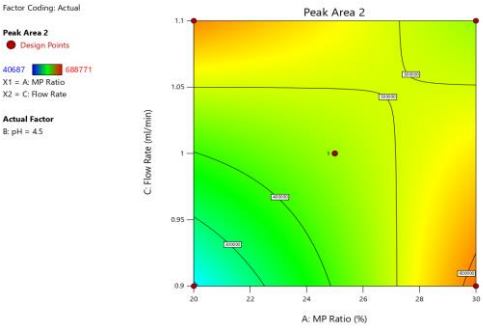
3B: Contour plot of peak area with pH as actual factor for valsartan



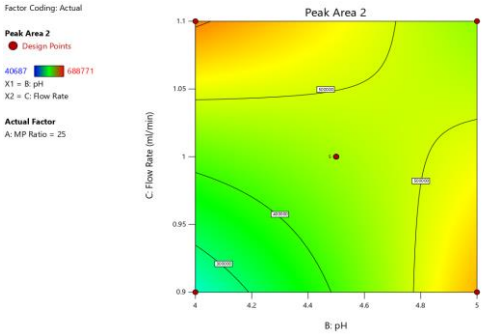
3C: Contour plot of peak area with MP Ratio as actual factor for valsartan



4A: Contour plot of peak area with Flow Rate as actual factor for sacubitril



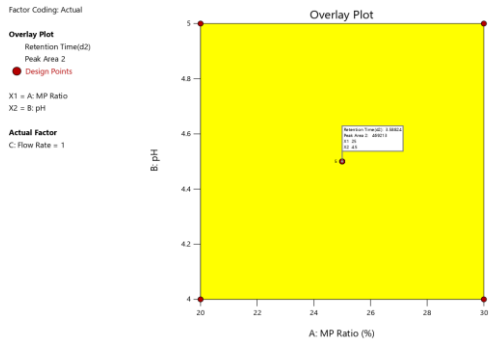
4B: Contour plot of peak area with pH as actual factor for sacubitril



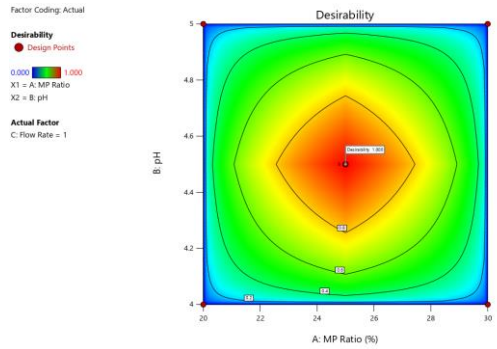
4C: Contour plot of peak area with MP Ratio as actual factor for sacubitril

Figure 2: Contour plots of valsartan and sacubitril for peak area

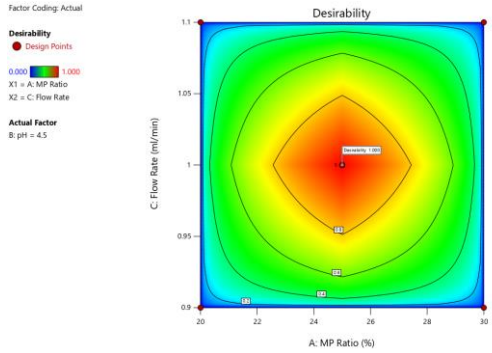
Overlay plot (fig. 3) depicts the optimal design space which interprets that if we use the optimized method obtained in the results of experimental design then we will get the results within the design space. It also shows that even if significant changes are made in CMPs we will obtain desired results.



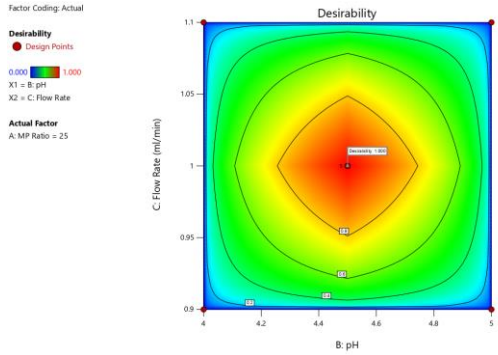
Overlay Plot



6A: Contour plot of Desirability with Flow Rate as actual factor



6B: Contour plot of Desirability with pH as actual factor



6C: Contour plot of Desirability with MP Ratio as actual factor

Figure 3: Overlay plot and Contour plots of Desirability

On the basis of assigned goals software determines the optimal chromatographic method with maximum desirability value which is 1. The desirability plots [fig. 3 (6A to 6C)] depicts that the optimized solution shows the desirability of 1. It also shows that significant changes in CMPs will still give desirability of 1.

Based on all trials of mobile phases using QbD approach Acetonitrile: Water (75:25) (v/v) pH 4.5 adjusted with formic acid was finalised. (Figure 4)

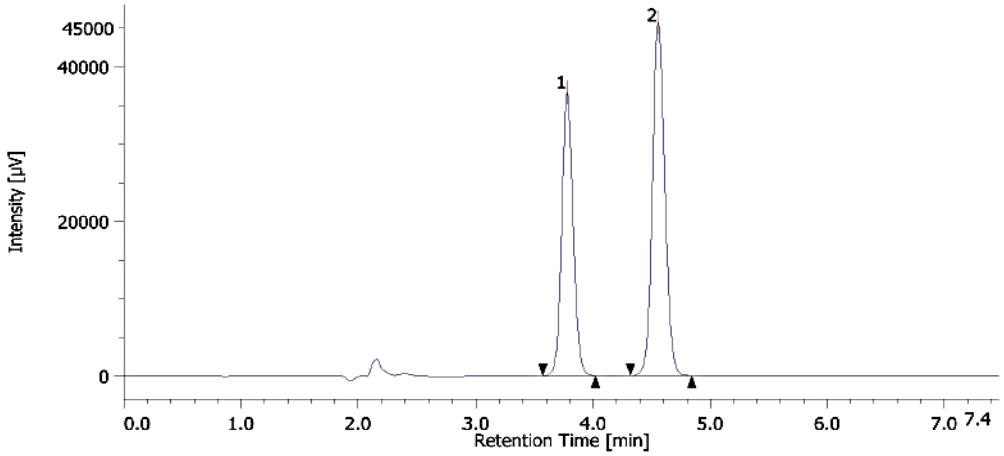


Figure 4: Chromatogram of Sacubitril and Valsartan (10ppm) using mobile phase as acetonitrile: water (75:25) (v/v) pH 4.5 adjusted with formic acid

Method Validation:

The results of linearity studies conducted on three consecutive days reveal that the method is linear over the ranges studied with high R^2 values and low intercepts. The results for intra-day and interday precision were all within the 2 % limit, indicating that the method is precise. Intra-day and inter-day precision results are summarized in Table no. 5. The accuracy of the method established by evaluating low, medium and high level concentrations of drugs in replicate (n=6) resulted in a mean percent recovery within ±15 % for both sacubitril and valsartan with % RSD values ranging from 0.13-0.20 % indicating that the method is accurate. The LOQ of the method established as described in the ICH Q2A guideline was 1 µg/ml with an associated % RSD of 1.79. By convention, the LOD was 0.3 µg/ml.

1. System Suitability:

As per guidelines tailing factor for the peaks of both drugs should not be more than 2.0.Theoretical plates should not be less than 2000. Resolution for the Sacubitril and Valsartan peaks in standard solution should not be less than 2. The results obtained are given in the Table no.4.

TABLE 4: RESULTS OF SYSTEM SUITABILITY

Parameters	**Valsartan**	**Sacubitril**
Tailing Factor	1.1	1.1
Theoretical Plates	7260	9108
Resolution	4.2	4.2

All the parameters passes their particular limits as per guidelines.

2. Specificity:

After the injecting LLOQ (0.5ppm) concentration blank run was taken. As blank run showed no peak at the retention time of both drugs it is proved that method is specific for the Valsartan and Sacubitril (Figure 5 and 6).

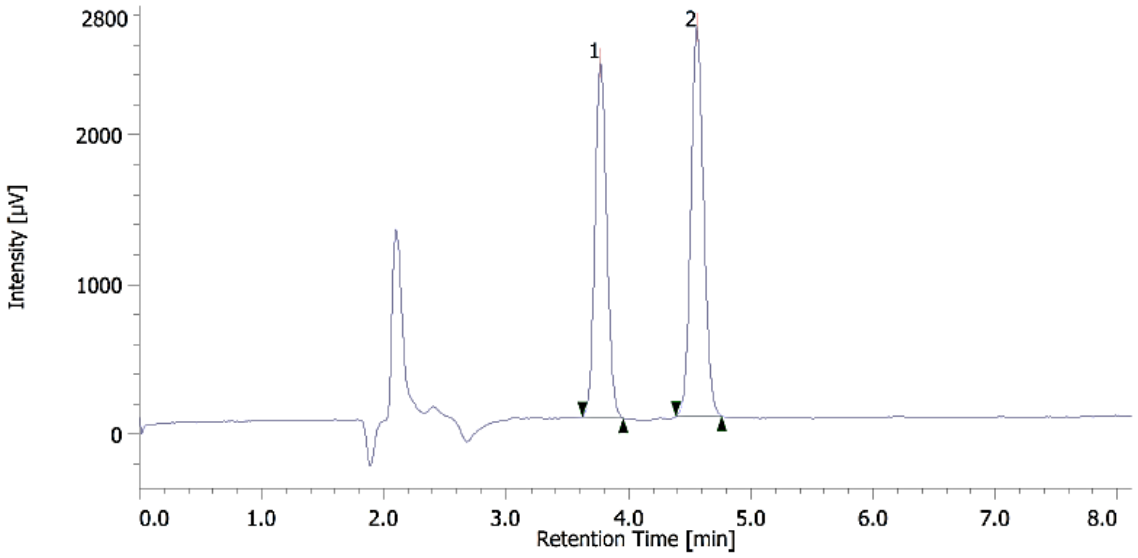


Figure 5: Chromatogram for specificity 0.5ppm

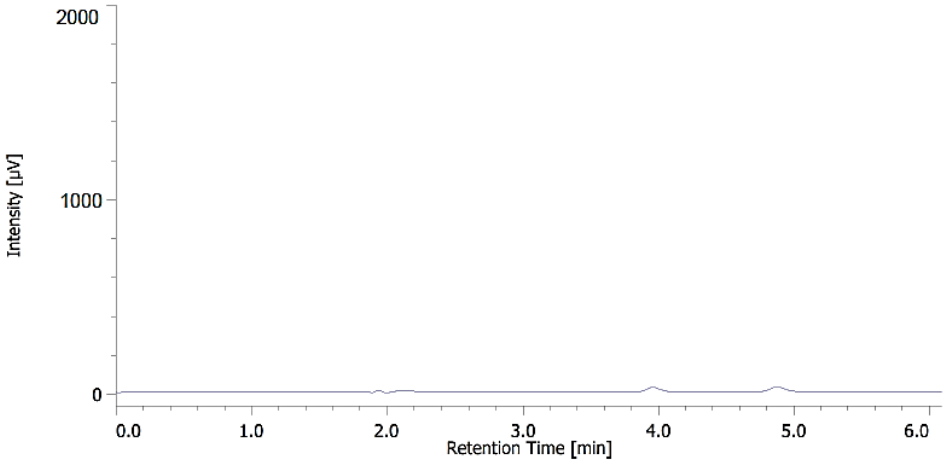


Figure 6: Chromatogram for specificity Blank run

3. Linearity:

Linearity was carried out in six different samples and these six samples were injected in triplicates. Concentration used for linearity were 0.5, 2, 4, 6, 8, 10 µg/ml.

For all three sets a calibration curve graph was plotted. The calibration curve of sacubitril and valsartan are given in Figure no. 7 and 8.

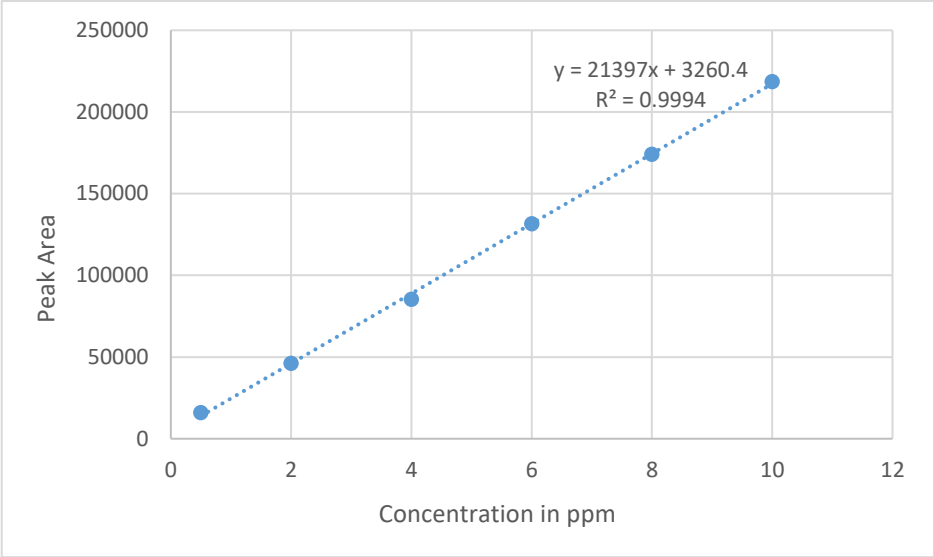


Figure 7: Calibration curve of valsartan

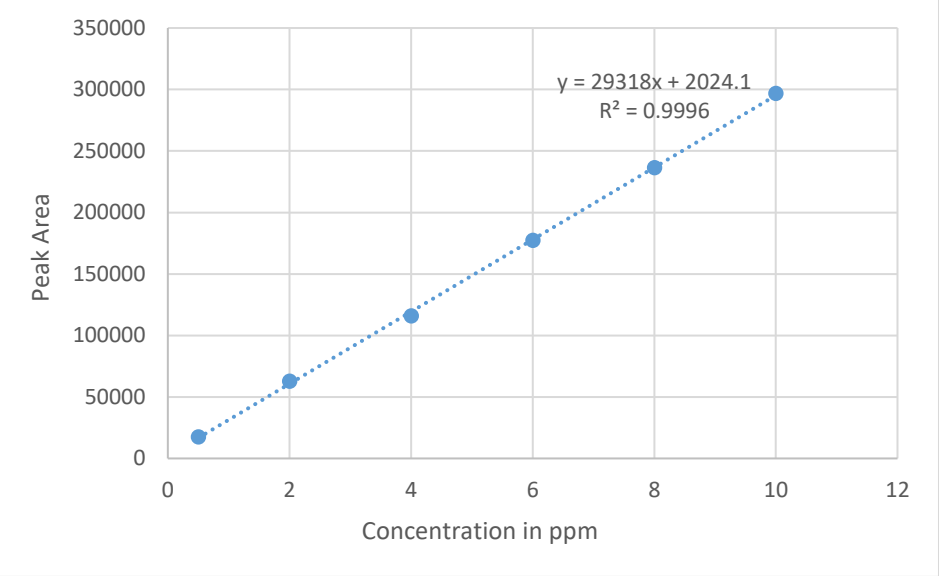


Figure 8: Calibration curve of sacubitril

Linear regression equation is as follows
$y = 21906.33x + 1986.037$ $R^2 = 0.998$ (Valsartan)
$y = 29938x + 293.533$ $R^2 = 0.998$ (Sacubitril)
Both the drugs showed linearity between concentration ranges of 0.5-10 ppm and had correlation coefficient of 0.998 with slope 21906.33 and y intercept 1986.037 for valsartan and correlation coefficient of 0.998 with slope 29938 and y intercept 293.533 for sacubitril for the graph of concentration against area.

4. Precision:
Precision indicates closeness of test results for six samples performed at three concentration levels. Intraday and Interday results are given in Table no.5. Results of precision study showed that the developed analytical method is precise.

The %RSD for both intraday and interday precision is below 2% for both drugs and thus we can predict that the developed method is precise.

TABLE 5: RESULTS OF PRECISION FOR VALSARTAN AND SACUBITRIL

	Valsartan	**Sacubitril**
Intraday	**%RSD**	**%RSD**
LQC (3ppm)	0.75	1.15
MQC (5ppm)	0.71	1.8
HQC (8ppm)	1.14	1.14
Interday		
LQC (3ppm)	0.53	0.58
MQC (5ppm)	0.91	1.02
HQC (8ppm)	1.53	1.78

5. Accuracy:

The demonstration of accuracy is mostly affected by how well systematic errors can be controlled. Results of accuracy are given in Table no.6 for valsartan and sacubitril respectively. Percent recovery should be in the range of 85% to 115%. Percent accuracy in this range indicates good accuracy of method.

The % recovery for both the drugs was found to be in the range of 85% - 115%. Thus the developed method is said to be accurate.

TABLE 6: RESULTS OF ACCURACY FOR VALSARTAN AND SACUBITRIL (n=4)

	Concentration Level	Nominal Concentration (µg/ml)	Average %Recovery	%RSD
Valsartan	50%	6	110.2	1.6
	100%	8	107.7	0.57
	150%	10	103.9	0.65
Sacubitril	50%	6	103.4	1.6
	100%	8	105.2	0.54
	150%	10	102.7	0.61

6. Limit of Detection and Limit of Quantitation:

LOD and LOQ is determined by calculation method

LOD = 3.3× σ/S

LOQ = 10× σ/S

Where, σ = standard deviation of y intercept and S = slope. The values for σ and S are obtained from the linear regression equation.

For Valsartan

LOD = 0.24 ppm

LOQ = 0.75 ppm

For Sacubitril

LOD = 0.24 ppm

LOQ = 0.74 ppm

The LOD and LOQ of valsartan was found to be 0.24 ppm and 0.75ppm respectively. The LOD and LOQ of sacubitril was found to be 0.24ppm and 0.74ppm respectively.

7. Stock Solution Stability:

After 24 hours in refrigerated conditions the solution gets turbid and the drugs in the solution gets precipitate out. When solution was used for analysis it altered the results as it is shown in chromatogram of Day 1 and Day 2 (Figure 9 and 10).

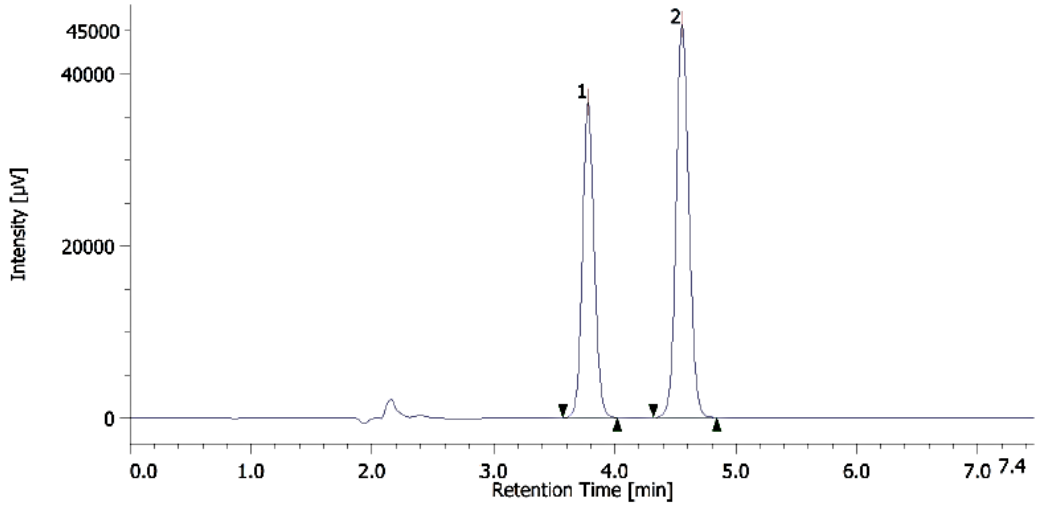


Figure 9: Chromatogram for Stock solution stability (10ppm) on Day 1

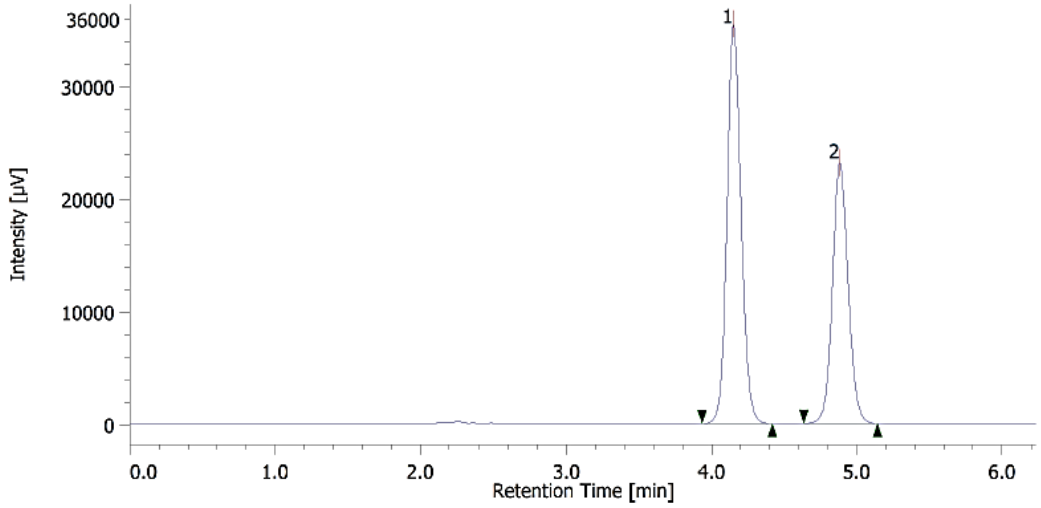


Figure 10: Chromatogram for Stock solution stability (10ppm) on Day 2

8. Robustness:

Results of robustness by changing flow rate, mobile phase composition and pH are shown in Table no.7. %RSD less than 2 indicates that the method is robust. On evaluation of the above results it can be concluded that variation in flow rate slightly affected the method but as %RSD is in range indicates that method is robust even after variation in flow rate.

On the other hand, variation in mobile phase ratio and pH did not affect the method and %RSD was also in range, so it indicates that method is robust even after variation in mobile phase ratio and pH. Hence it can be concluded that the method is robust even in varied parameters.

TABLE 7: ROBUSTNESS DATA OF VALSARTAN AND SACUBITRIL (N=3)

Parameter		**Valsartan**			**Sacubitril**		
		Area	**SD**	**%RSD**	**Area**	**SD**	**%RSD**
Flow Rate	0.9	127012.7	1151.398	0.9	151060.3	1287.356	0.85
	1.1	112283	341.5889	0.3	134490	215.6687	0.16
MP Composition	-2%	117752.7	273.4343	0.23	142640.3	723.1994	0.50
	+2%	121479.7	547.658	0.45	145817.7	717.8066	0.49
pH	4.4	136851.7	2249.922	1.6	165719	2650.78	1.5
	4.6	136933	1905.935	1.3	165886.3	2365.862	1.4

Assay:

Tablet brand name Azmarda from Cipla which have label claim of 200 mg, 103 mg of valsartan and 97 mg of sacubitril.

The amount of valsartan in given tablet obtained was 103.8 mg and the amount of sacubitril obtained was 96.08mg. Thus % Assay was found to be 100.7% w/w and 99.05% w/w for valsartan and sacubitril respectively.

In conclusion, current study provides a simple, accurate and precise HPLC method optimized by the 'Quality by design' approach for simultaneous estimation of Valsartan and Sacubitril. The method is validated as per ICH guidelines and passed all validation criterions as per ICH guidelines. The method can be used for simultaneous estimation of Valsartan and Sacubitril in bulk drug and formulation using High Performance Liquid Chromatography.

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