RESEARCH PAPER

Development & Validation of Reversed-Phase Ultra-Fast Liquid Chromatography Method for Simultaneous Estimation of Anti-Hypertensive Drugs in Their Dosage Form

Prafulla Chaudhari[1], Priyanka Chaudhari[2]*, Rohini Kolhe[3], Prashant Ghode[4], Asawari Pachauri[5], Madhavi Bindu[1]

1. Department of Pharmaceutics, Rajarshi Shahu College of Pharmacy and Research, Tathawade, Pune, Maharashtra, India.
2. *Department of Pharmaceutical Chemistry, SJVPM's, Rasiklal M. Dhariwal Institute of Pharmaceutical Education and Research, Chinchwad, Pune, Maharashtra, India.
3. Department of Pharmacognosy, Rajarshi Shahu College of Pharmacy and Research, Tathawade, Pune, Maharashtra, India.
4. Department of Pharmaceutical Chemistry, TSSM's Jayawant Institute of Pharmacy and Research, Bavdhan, Pune, Maharashtra, India.
5. Department of Pharmaceutical Chemistry, Rajarshi Shahu College of Pharmacy and Research, Tathawade, Pune, Maharashtra, India.

Corresponding Author* Dr. Priyanka S. Chaudhari
Associate Professor & HOD Department of Pharmaceutical Chemistry SJVPM's, Rasiklal M. Dhariwal Institute of Pharmaceutical Education and Research, Chinchwad,Pune, Maharashtra, India. Email: psc.rmdiper@gmail.com

Abstract
A simple, sensitive, precise, accurate, and stability-indicating reverse phase ultra-fast liquid chromatography (RP-UFLC) method was developed and validated for the simultaneous estimation of Fimasartan potassium trihydrate, Valsartan, and Sacubitril in pharmaceutical dosage forms. Chromatographic separation was achieved on a Shimadzu Shim-pack C18 column (250 × 4.6 mm, 5 µm) using a gradient elution system comprising solvent A [methanol:water:0.1% orthophosphoric acid (70:10:20, v/v/v)] and solvent B [methanol:0.1% orthophosphoric acid (80:20, v/v)] at a flow rate of 1.0 mL/min. Detection was performed using a PDA detector at 258 nm, with a total run time of 15 min. The developed method was validated in accordance with ICH Q2(R1) guidelines and exhibited excellent linearity over the selected concentration ranges, with correlation coefficients (r^2) of 0.9997, 0.9997, and 0.9996 for Fimasartan potassium trihydrate, Valsartan, and Sacubitril, respectively. The method demonstrated satisfactory precision with %RSD values below 2%, accuracy with recoveries within the acceptable range of 98–102%, and adequate sensitivity as confirmed by low limits of detection (LOD) and quantification (LOQ). Furthermore, the method was found to be specific, robust, and suitable for routine quality control analysis without interference from excipients. Therefore, the proposed RP-UFLC method is reliable, economical, and applicable for the simultaneous qualitative and quantitative estimation of Fimasartan potassium trihydrate, Valsartan, and Sacubitril in bulk drugs and pharmaceutical dosage forms.

Keywords: Fimasartan potassium trihydrate, Valsartan, Sacubitril, Method development, RP-UFLC.

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1. Introduction

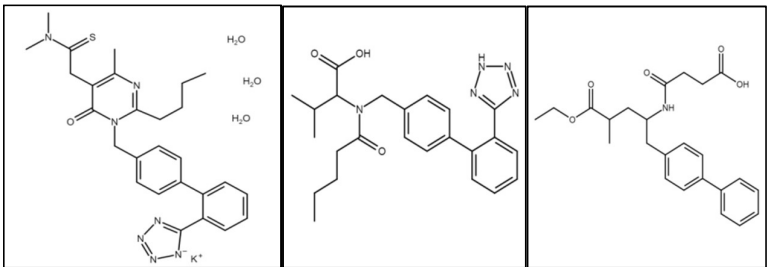
Fimasartan potassium trihydrate (FIMA), is chemically known as potassium {(2R)-2-{[4′-(2-oxo-4,5-dihydro-1,3-oxazol-5-yl)biphenyl-4-yl]methyl}-2H-1,2,3,4-tetrazol-5-yl}methylimidazole-5-carboxylate trihydrate having molecular formula $C_{27}H_{36}KN_7O_4S$ and molecular weight 593.8 g/mol. Fimasartan is an angiotensin II receptor antagonist (ARB) drug employed in the treatment of both hypertension and heart failure[1]. Valsartan (VAL) is chemically known as (2S)-3-methyl-2-[pentanoyl-[[4-[2-(2H-tetrazol-5-yl) phenyl]phenyl]methyl]amino] butanoic acid having molecular formula $C_{24}H_{29}N_5O_3$ and molecular weight 435.5 g/mol. Valsartan belongs to the angiotensin II receptor blocker (ARB). Valsartan is indicated for the treatment of hypertension to reduce the risk of fatal and non-fatal cardiovascular events, primarily strokes and myocardial infarctions[2–4].

Sacubitril (SAC) is chemically known as 4-[[(2S,4R)-5-ethoxy-4-methyl-5-oxo-1-(4-phenylphenyl)pentan-2-yl]amino]-4-oxobutanoic acid having molecular formula $C_{24}H_{29}NO_5$ and molecular weight 411.5 g/mol. Sacubitril is a prodrug neprilysin inhibitor used in combination with valsartan to reduce the risk of cardiovascular events in patients with chronic heart

*Author for Correspondence: psc.rmdiper@gmail.com

failure (NYHA Class II-IV) and reduced ejection fraction[5,6].

It can be challenging to simultaneously determine three or more compounds using liquid chromatography, and the challenge is made worse if these compounds are present in pharmaceutical dosage forms where excipients may interfere. A literature survey revealed that a combination FIMA[7–14], VAL and SAC for its determination in alone or in combination with other drug were available FIMA, VAL[15–26] and SAC[27–36]. But there is no method available for the simultaneous measurement of FIMA, VAL and SAC. As a result, the purpose of the current study is to simultaneously estimate FIMA, VAL and SAC in bulk and synthetic mixtures using the RP-UFLC method.Compared to HPTLC, RP-UFLC is more affordable and very sensitive. It is compatible with every significant HPLC detector, including the photodiode array (PDA), UV/Visible, and Refractive Index detectors[37].



(A) (B) (C)

Figure 1: Chemical structure of (A) Fimasartan potassium trihydrate, (B) Valsartan and (C) Sacubitril.

2. Material and Methods

2.1 Material

Fimasartan (FIMA), Valsartan (VAL), and Sacubitril (SAC) were kindly supplied as gift samples by Cipla Pvt. Ltd., Mumbai, India. HPLC-grade acetonitrile, methanol, and water were obtained from Merck (LiChrosolv®, Mumbai, India). All other chemicals and reagents used in the study were of analytical reagent (AR) grade. Ortho phosphoric acid was obtained from Research-Lab Fine Chem Industries, Mumbai, India. Prior to use, mobile phase was filtered through a 0.22 µm membrane filter (Millipore, Bedford, MA, USA) and the solutions were filtered using Filter paper of 11 µm (Whatman). The pharmaceutical preparations of the investigated compounds Fimanta (60 mg Fimasartan potassium trihydrate) and CIDMUS 50 (26 mg of Valsartan and 24 mg of Sacubitril) were obtained from the local medical store.

2.2 Chromatographic condition-UFLC System

The chromatographic analysis was performed using a Shimadzu UFLC system (Model No. LC-20AD, Shimadzu Corporation, Kyoto, Japan) equipped with a tertiary solvent delivery system, a Rheodyne injector fitted with a 20 µL sample loop, and a photodiode array (PDA) detector. Data acquisition and processing were carried out using LabSolutions software[38]. Chromatographic separation was achieved gradiently at room temperature on the Shim-pack C_{18} column with the dimensions 4.6 mm I.D, 250 mm length, and 5 µm particle size. The mobile phases used for the gradient type analysis were mobile phase A consisting of methanol: water: 0.1% OPA (70: 10: 20 v/v/v) and mobile phase B consisting of methanol: 0.1% OPA (80: 20 v/v) with a flow rate of 1.0 mL/min.

2.3 Preparation of standard solution

A standard stock solution was prepared by accurately weighing and dissolving 60 mg of Fimasartan (FIMA), 26 mg of Valsartan (VAL), and 24 mg of Sacubitril (SAC) in a few millilitres of methanol in a 25.0 mL volumetric flask. The volume was then made up to the mark with methanol. Subsequently, 2.0 mL of the resulting stock solution was transferred into another 25.0 mL volumetric flask, and the volume was adjusted to the mark with methanol to obtain the working standard solution.

Aliquots of 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the working standard solution were transferred into five separate 10.0 mL volumetric flasks, and the volume in each flask was made up to the mark with methanol. The resulting solutions contained FIMA at concentrations of 19.2, 38.4, 57.6, 76.8, and 96.0 µg/mL; VAL at concentrations of 8.32, 16.64, 24.96, 33.28, and 41.60 µg/mL; and SAC at concentrations of 7.68, 15.36, 23.04, 30.72, and 38.40 µg/mL, respectively[39].

2.4 Preparation of sample solution

Ten tablets of each drug were taken and crushed into powder with the help of mortar and pestle and tablet powder equivalent to 60 mg of FIMA, 26 mg of VAL and 24 mg of SAC were weighed and transferred to 25.0mL of volumetric flask containing 10.0mL of methanol and then sonicate this solution for 15 minutes. After this make the volume up to the mark with methanol. Filter the solution with whatman filter paper. The resulting solution was filtered through a 0.45 µm membrane filter. An aliquot of 2.0 mL of the filtered solution was transferred into a 25.0 mL volumetric flask, and the volume was made up to the mark with methanol. Subsequently, 3.0 mL of this solution was transferred into a 10.0 mL volumetric flask, and the volume was adjusted to the mark with methanol to obtain final concentrations of 57.6 µg/mL

of Fimasartan (FIMA), 24.96 µg/mL of Valsartan (VAL), and 23.04 µg/mL of Sacubitril (SAC). A 20 µL aliquot of the final solution was injected into the chromatographic system under the optimized chromatographic conditions, and the percentage assay of FIMA, VAL, and SAC was determined using the proposed RP-UFLC method[40].

3. Validation

Validation of the proposed method was done according to International Council on Harmonization (ICH) guidelines Q2 (R1) for all parameters[41].

3.1 System Suitability

The system suitability testingis crucial for ensuring the high performance of the chromatographic system. The system suitability parameters like resolution, theoretical plates and tailing factor were calculated and compared with standard values[42].

3.2 Linearity

The linearity of the method was studied in combination using a standard solution with concentrations of 19.2-96µg/mL for FIMA, 8.32-41.60µg/mL for VAL and 7.68-38.4µg/mL for SAC. The calibration range was made in such a way that the ratio of combination was maintained throughout the analysis. The calibration curves were established by plotting the peak areas against the corresponding concentrations[43].

3.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined utilizing the linearity data and are calculated using below formula.

LOD= 3.3× Standard deviation/ slope

LOQ= 10× Standard deviation/ slope

3.4 Accuracy

The accuracy of the proposed method was evaluated by the standard addition technique. Known amounts of the standard drugs corresponding to 80%, 100% and 120% of the pre-quantified synthetic mixture sample were added to the sample solution. The pre-quantified sample solution contained 57.6 µg/mL of Fimasartan (FIMA), 24.96 µg/mL of Valsartan (VAL) and 23.04 µg/mL of Sacubitril (SAC). Standard additions equivalent to 46.08, 57.60, and 69.12 µg/mL of FIMA; 19.97, 24.96, and 29.95 µg/mL of VAL; and 18.43, 23.04, and 27.65 µg/mL of SAC**,** representing the 80%, 100%, and 120% levels, respectively, were spiked into the sample solution. Each level was analyzed in triplicate and the percentage recoveries were calculated to assess the accuracy of the proposed method[44].

3.5 Precision

Precision of the proposed method was evaluated in terms of system precision, intra-day precision, and inter-day precision. System precision was assessed by six replicate injections of a standard solution containing 57.6 µg/mL of Fimasartan (FIMA), 24.96 µg/mL of Valsartan (VAL), and 23.04 µg/mL of Sacubitril (SAC)**.** Intra-day precision (repeatability) was determined by analyzing three different concentration levels, namely 38.4, 57.6, and 76.8 µg/mL of FIMA; 16.64, 24.96, and 33.28 µg/mL of VAL; and 15.36, 23.04, and 30.72 µg/mL of SAC, each in triplicate on the same day. Inter-day precision (intermediate precision) was evaluated by analyzing the same three concentration levels in triplicate on three consecutive days. The precision of the method was expressed as the percentage relative standard deviation (%RSD) of the measured responses. The mean peak areas, SD and % RSD values were calculated for system precision, intra-day precision and inter-day precision[44]. The data of precision studies by the proposed RP-UFLC method are shown in Table 4.

3.6 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the method was ascertained by analyzing sample solutions. The bands of FIMA, VAL and SAC were confirmed by comparing retention time values and relative spectra of samples with those of standards. Specificity is done for identifying interference in the drug and excipient while analysis. Using LabSolution software, there is no need to analyse and identify the separate solutions of individual drugs because it simultaneously determinesthe UV of the compounds[44].

3.7 Robustness

According to ICH guidelines, robustness has to be performed to understand the effect of slight changes on the experimental conditions for the newly proposed method. A method is considered robust if it is unaffected by minor adjustments to the operating environment[44]. Robustness of the method was studied by change in flow rate at 1.0 ± 0.1 mL/min, change in wavelength at 258 ± 2 nm and change in mobile phase ratio for the analysis. The data of robustness studies by the proposed RP-UFLC method are shown in Table 5.

4. RESULTS AND DISCUSSION

4.1 Method Development

The objective of this present work was to resolve the chromatographic peaks for the three drugs (FIMA, VAL and SAC). Various solvents were used for the resolution such as water, acetonitrile, methanol and 0.1 % ortho-phosphoric acid. Firstly, the trials were taken using isocratic mode on various mobile phase compositions. Methanol: Acetonitrile: 0.1% OPA (40:40:20 v/v/v) was used isocratically but there was no resolution for FIMA and VAL. After this concentration of acetonitrile was decreased to see the effect on resolution but the peaks did not resolve. So, Methanol: 0.1% OPA was given isocratically the peaks were getting resolved as compared to earlier compositions. After this, the concentrations were varied for the mixture of Methanol: 0.1% OPA but there was no better resolution seen. At the last, Methanol: 0.1% OPA: water (70:20:10 v/v/v) was used

to achieve better resolution for all three drugs but the retention time of Sacubitril was too late at 17.6 min. So by applying gradient mode the retention time of Sacubitril was achieved at 13.5 min. The optimized trial was of gradient mode using mobile phase A i.e., Methanol: 0.1% OPA: water (70:20:10 v/v/v) and mobile phase B i.e., Methanol: 0.1% OPA (80:20 v/v) at flow rate of 1.0 mL/min. The retention time for FIMA, VAL and SAC were achieved at 8.5 min, 9.6 min and 13.5 min respectively and were detected at 258 nm.

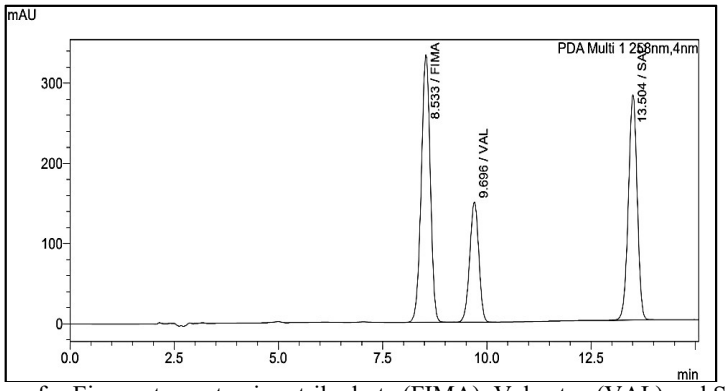


Figure 2: Chromatogram for Fimasartan potassium trihydrate (FIMA), Valsartan (VAL) and Sacubitril (SAC) using gradient mode.

4.2 Method Validation

System Suitability tests

The system suitability tests were carried out on freshly prepared standard solutions of FIMA (19.2 µg/mL), VAL (8.32 µg/mL) and SAC (7.68 µg/mL). The system suitability data of the proposed RP-UFLC method for FIMA, VAL and SAC are shown in Table 1.

Table 1: System suitability data for FIMA, VAL and SAC [Proposed RP-UFLC method].

Parameters	FIMA	VAL	SAC
Retention time (min)	8.5	9.6	13.5
Tailing Factor	0.932	0.959	0.948
Theoretical Factor	6514	8381	16308
Resolution	0.00	2.709	9.121

Linearity

Linearity was established by plotting a graph of the concentrations of the analytes against the peak areas. The statistical data of regression analysis of the calibration curves for FIMA, VAL and SAC by the RP-UFLC method are shown in Table 2. Linearity with a good regression coefficient was achieved in the concentration of 19.2-96 µg/mL for FIMA, 8.32-41.60 µg/mL for VAL and 7.68-38.4 µg/mL for SAC. Limit of detection (LOD) and limit of quantification (LOQ) were calculated using linearity data.

Table 2. Statistical Parameters of the Calibration Curves for Fimasartan (FIMA), Valsartan (VAL), and Sacubitril (SAC) Obtained by the Proposed RP-UFLC Method

Parameters	FIMA	VAL	SAC
Range (µg/mL)	19.2 – 96	8.32 - 41.60	7.68 - 38.4
Regression coefficient (r^2)	0.9997	0.9997	0.9996
Slope	47926	22654	44140
Intercept	22582	4066.9	15370
Limit of Detection (LOD)	3.83	1.73	1.81
Limit of Quantification (LOQ)	11.62	5.24	5.49

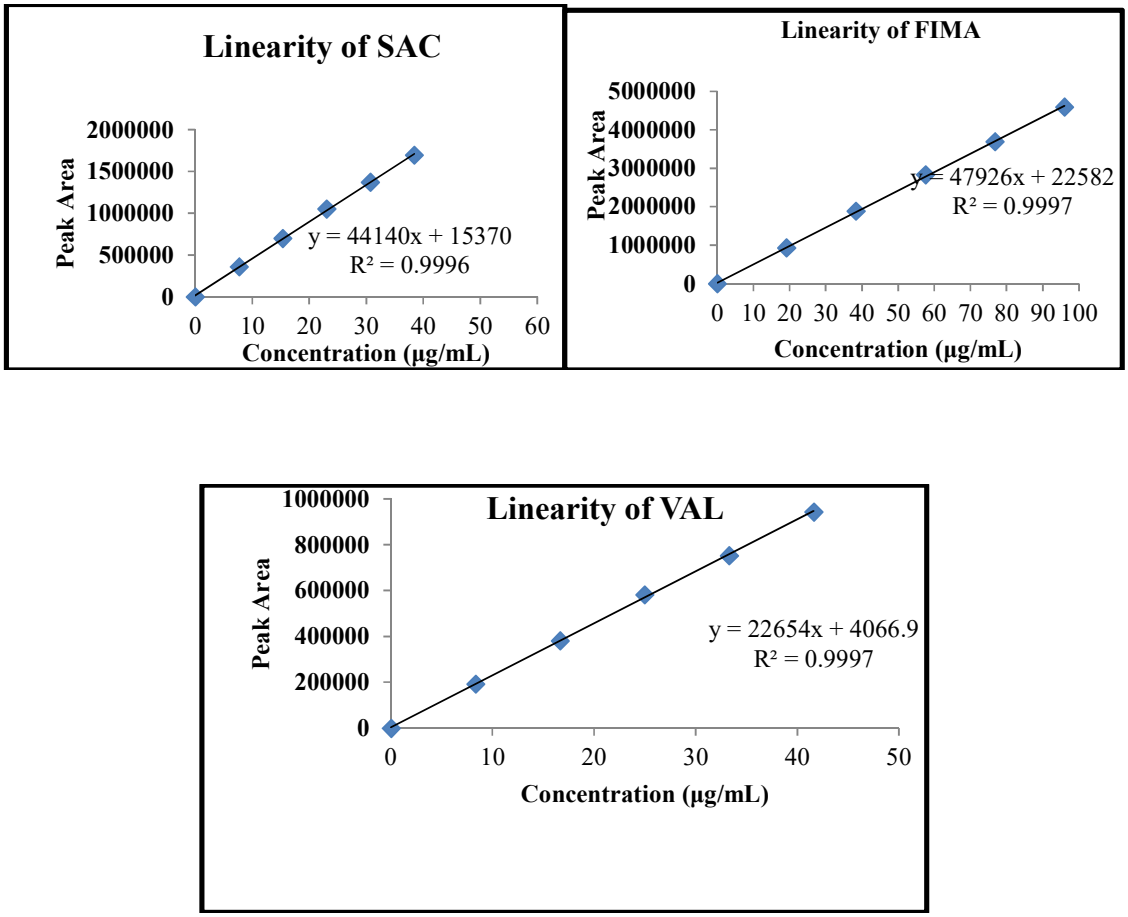


Figure 3: Calibration curves for FIMA, VAL and SAC.

Accuracy

In order to confirm the accuracy of the proposed method, recovery studies using the standard addition method were carried out. FIMA (57.60 µg/mL), VAL (24.96 µg/mL) and SAC (23.04 µg/mL), previously assessed sample solution, were spiked with 80%, 100% and 120% additional standards of FIMA, VAL and SAC respectively and the resultant mixture were examined using the proposed method. The mean of percent recovery was found to be 99.86% for FIMA, 99.77% for VAL and 99.85% for SAC. The low percent relative standard deviation confirmed that the proposed method is accurate. The data for the accuracy studies of the proposed method is shown in Table 3.

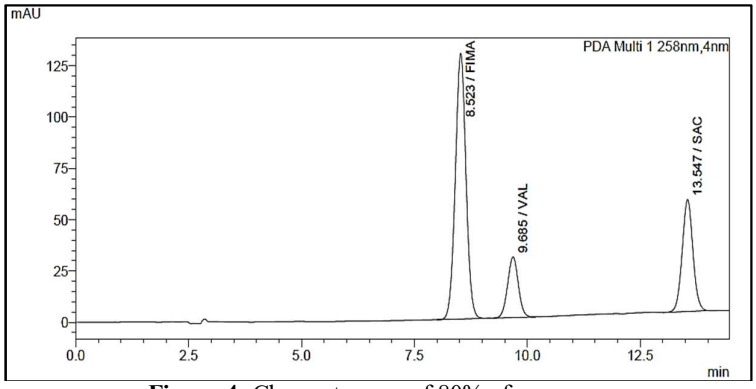


Figure 4: Chromatogram of 80% of accuracy

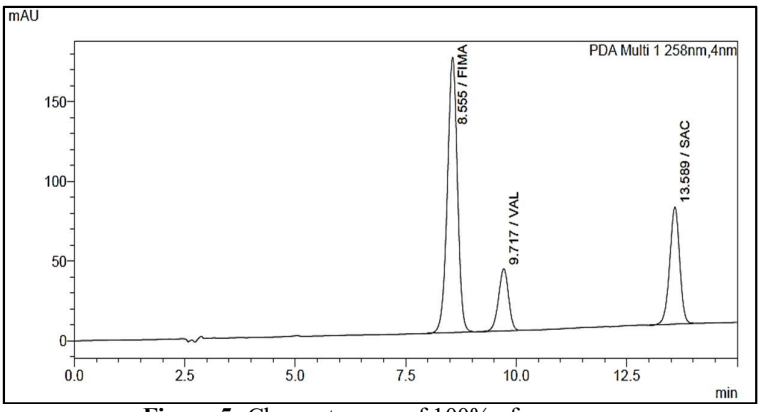


Figure 5: Chromatogram of 100% of accuracy

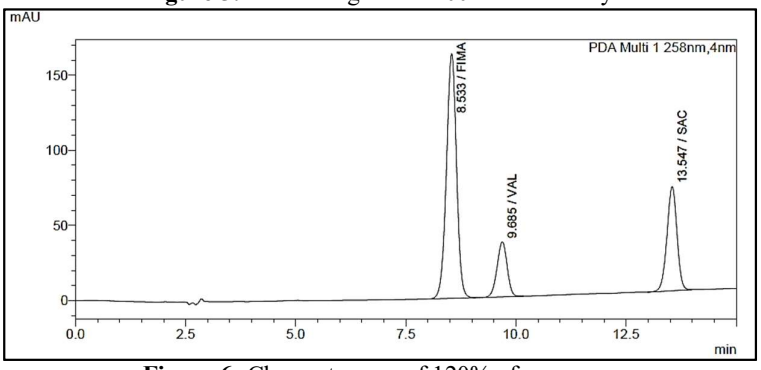


Figure 6: Chromatogram of 120% of accuracy

Table 3: Data of accuracy studies of the proposed RP-UFLC method.

Drug	Level	Mean Peak Area ± SD	% Recovery	% RSD
FIMA	80%	2644085.33 ± 4582.04	99.54	0.389
	100%	2954413 ± 4407.91	100.06	0.296
	120%	3250951.33 ± 2185.48	99.99	0.122
VAL	80%	543023.33 ± 423.13	99.49	0.174
	100%	601269.67 ± 892.09	100.24	0.295
	120%	659386.33 ± 1209.90	99.58	0.336
SAC	80%	991501.33 ± 793.65	99.62	0.182
	100%	1104951.33 ± 3269.96	100.14	0.595
	120%	1210272 ± 2683.45	99.78	0.410

Precision

System precision was determined by analyzing the sample solution of middle concentrations of three drugs by injecting six times consequently. Intra-day and inter-day precisions were determined by analyzing the standard solution of the middle concentrations of three drugs for three times a day and for three consecutive days respectively. The low % RSD confirms the proposed method is precise.The % RSD were calculated and are given in Table 4.

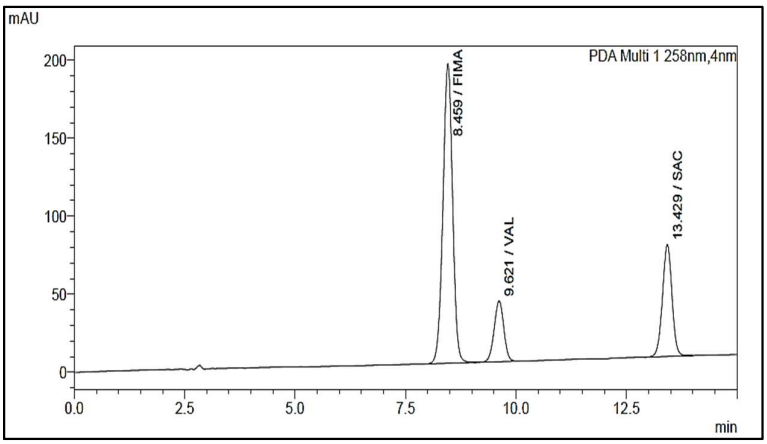


Figure 7: Representative chromatogram of system precision of drugs.

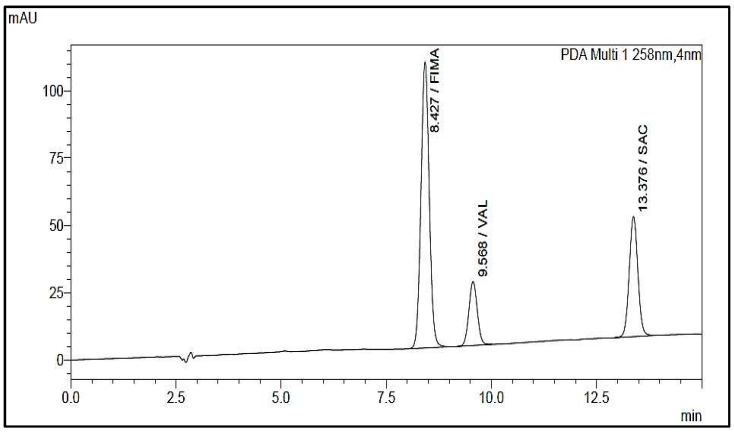


Figure 8: Representative chromatogram of intra-day precision of drugs.

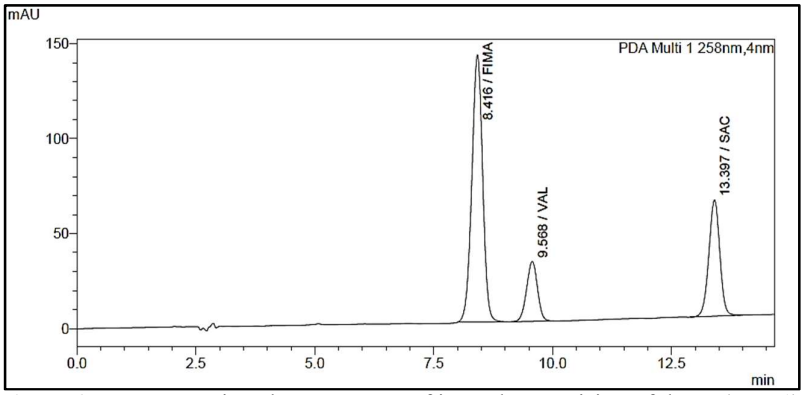


Figure 9: Representative chromatogram of inter-day precision of drugs (Day-1).

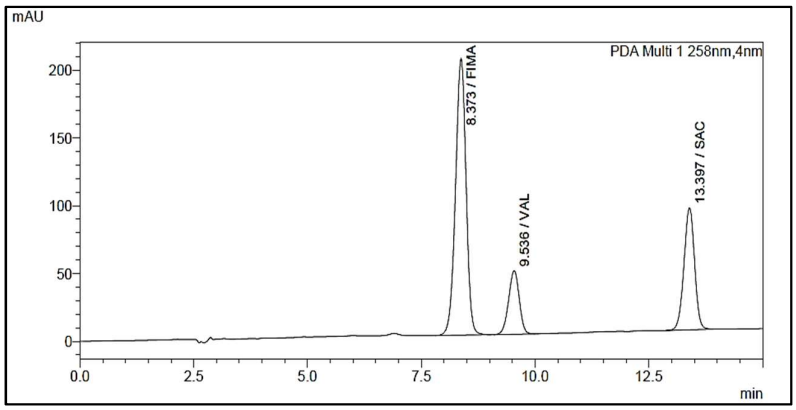


Figure 10: Representative chromatogram of inter-day precision of drugs (Day-2).

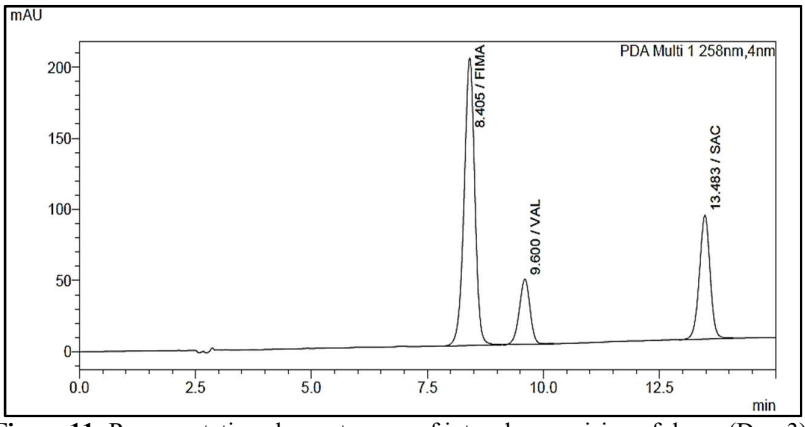


Figure 11: Representative chromatogram of inter-day precision of drugs (Day-3).

Table 4: Data of precision studies for the proposed RP-UFLC method

Drug	System Precision		Intra-day Precision		Inter-day Precision	
	Mean Peak Area ± SD (n=6)	**% RSD**	**Mean Peak Area ± SD (n=3)**	**% RSD**	**Mean Peak Area ± SD (n=3)**	**% RSD**
FIMA	2845671.17 ± 13172.42	0.463	3012200.33 ± 7576.50	0.252	3052007 ± 62372.15	0.463
VAL	584505.8333 ± 3207.41	0.549	614381.83 ± 3506.22	0.571	611205 ± 5139.34	0.516
SAC	1056754.833 ± 3698.78	0.350	1136500.83 ± 5466.35	0.481	1141409.67 ± 6779.99	0.439

Specificity

In the run time, no interfering peaks were discovered in the chromatogram of the formulation, proving that the excipients employed in pharmaceutical formulations did not interfere with the proposed method, which indicates that the method was specific.

Robustness

The robustness of the proposed method was determined by small changes in different parameters such as flow rate 1.0 ± 0.1 mL/min, wavelength 258 ± 2 nm and mobile phase composition ± 0.2% which did not significantly affect the resolution between the drugs eluted. The low % RSD values confirm that the proposed method is robust.

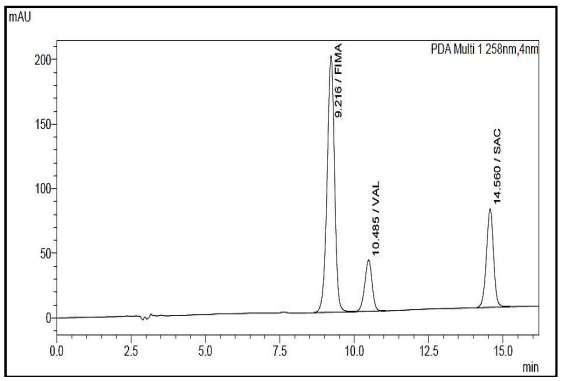


Figure 12: Representative chromatogram of flow rate at 0.9 mL/min.

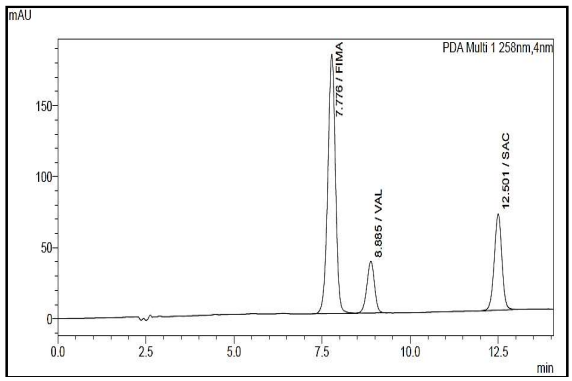


Figure 13: Representative chromatogram of flow rate at 1.1 mL/min.

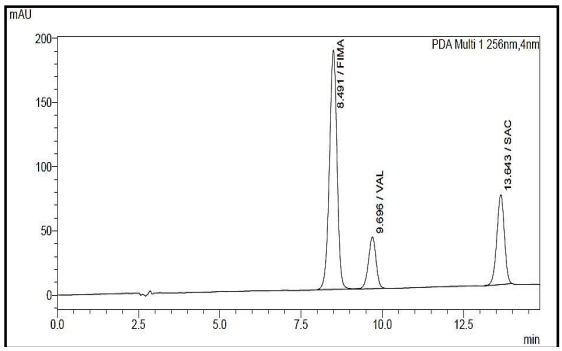


Figure 14: Representative chromatogram at wavelength 256 nm.

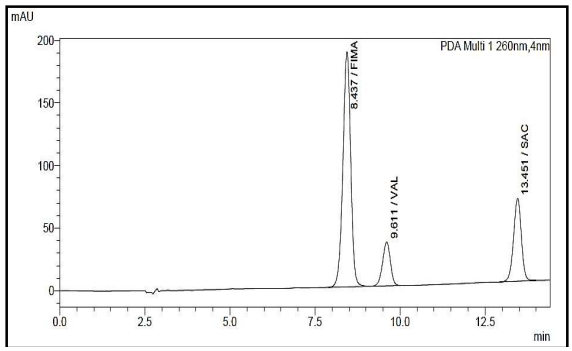


Figure 15: Representative chromatogram at wavelength 260 nm.

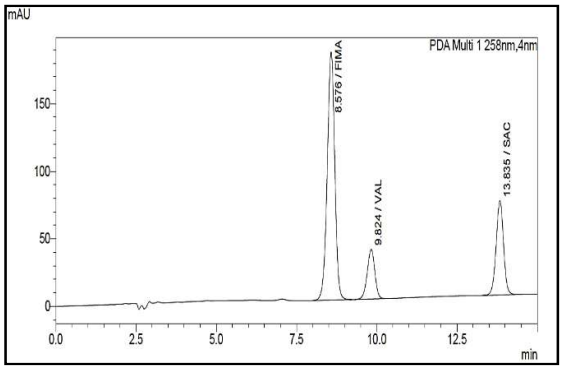


Figure 16: Chromatogram of mobile phase composition [Methanol: 0.1% OPA: Water (70.20:20:9.80 v/v/v)]

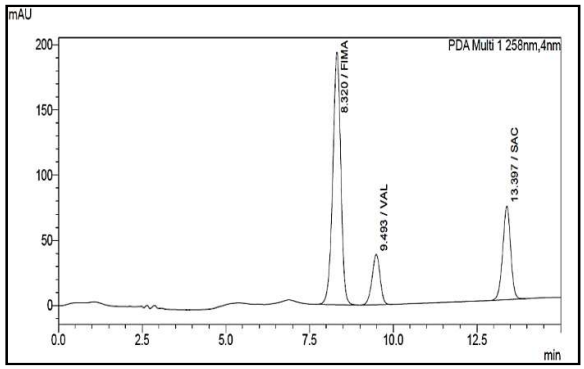


Figure 17: Chromatogram of mobile phase composition [Methanol: 0.1% OPA: Water (70.20: 19.80: 10 v/v/v)]

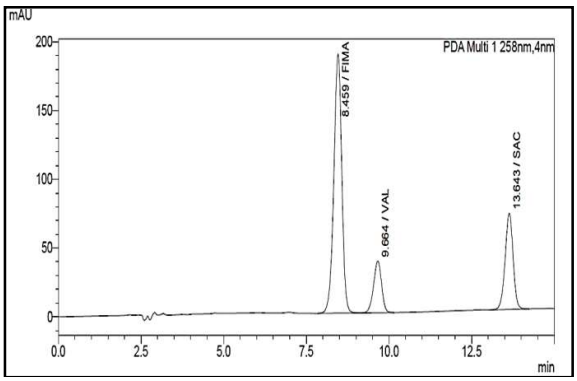


Figure 18: Chromatogram of mobile phase composition

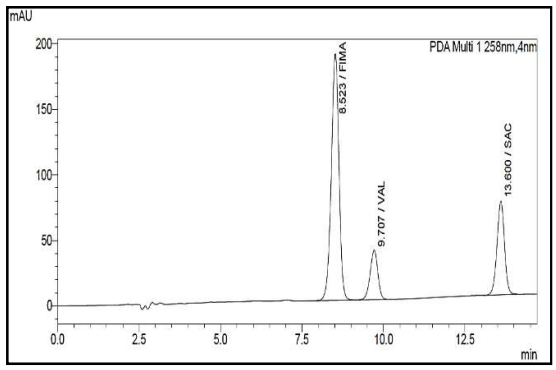


Figure 19: Chromatogram of mobile phase composition

[Methanol: 0.1% OPA: Water (70: 20.20: 9.80 v/v/v)]

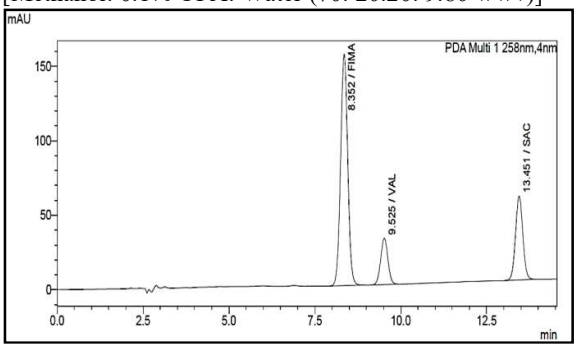


Figure 20: Chromatogram of mobile phase composition [Methanol: 0.1% OPA: Water (70: 19.80: 10.20 v/v/v)]

[Methanol: 0.1% OPA: Water (69.80: 20.20: 10 v/v/v)]

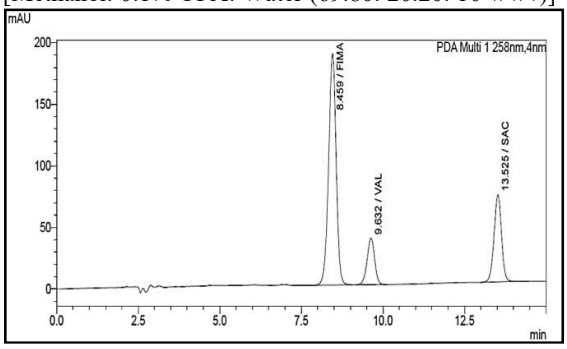


Figure 21: Chromatogram of mobile phase composition [Methanol: 0.1% OPA: Water (69.80: 20: 10.20 v/v/v)]

Table 5: Robustness data of the proposed method

Parameters	Peak Area ± %RSD		
	FIMA	**VAL**	**SAC**
Flow rate			
0.9 mL/min (Low)	3126214 ± 0.45	622610.5 ± 0.15	1146240 ± 1.44
1.0 mL/min (Normal)	3093703 ± 0.78	621308.5 ± 0.62	1139727 ± 0.61
1.1 mL/min (High)	3036855 ± 1.76	610016.5 ± 0.17	1128458 ± 1.41
Wavelength			
256 nm (Low)	3038347 ± 0.43	615737 ± 0.21	1140297 ± 0.97
258 nm (Normal)	3058219 ± 0.29	615282 ± 0.20	1135714 ± 0.83
260 nm (High)	3098531 ± 0.12	611372.5 ± 0.15	1132037 ± 0.90
Mobile phase composition			
Methanol: 0.1% OPA: Water (70: 20: 10 v/v/v) (Normal)	3073020 ± 0.18	617245 ± 0.30	1135679 ± 0.87
Methanol: 0.1% OPA: Water (70.20: 20: 9.80 v/v/v	3076832 ± 0.62	611852 ± 0.78	1132643 ± 0.97
Methanol: 0.1% OPA: Water (70.20: 19.80: 10 v/v/v	3090045 ± 0.82	616350.5 ± 0.62	1152863 ± 0.96
Methanol: 0.1% OPA: Water (70: 20.20: 9.80 v/v/v	3109612 ± 0.66	615269 ± 0.17	1133210 ± 1.11
Methanol: 0.1% OPA: Water (69.80: 20.20: 10 v/v/v	3065373 ± 0.28	622911.5 ± 0.09	1133684 ± 1.04
Methanol: 0.1% OPA: Water (70: 19.80: 10.20 v/v/v	3044696 ± 0.20	618408 ± 0.69	1131647 ± 0.74
Methanol: 0.1% OPA: Water (69.80: 20: 10.20 v/v/v	3038539 ± 0.61	613430 ± 0.42	1129654 ± 0.58

5. Analysis of pharmaceutical preparations

The proposed method of RP-UFLC was applied for the determination of FIMA, VAL and SAC in their formulation. The assay of the formulation was taken in combination by injecting six times of the sample solution of the middle concentrations of calibration curve. The results of the assay of the tablet formulation Fimanta (60 mg Fimasartan potassium trihydrate) and CIDMUS 50 (26 mg of Valsartan and 24 mg of Sacubitril) were found to be in the range of 99-101%. The data of assay studies of the formulation are given below in Table 6.

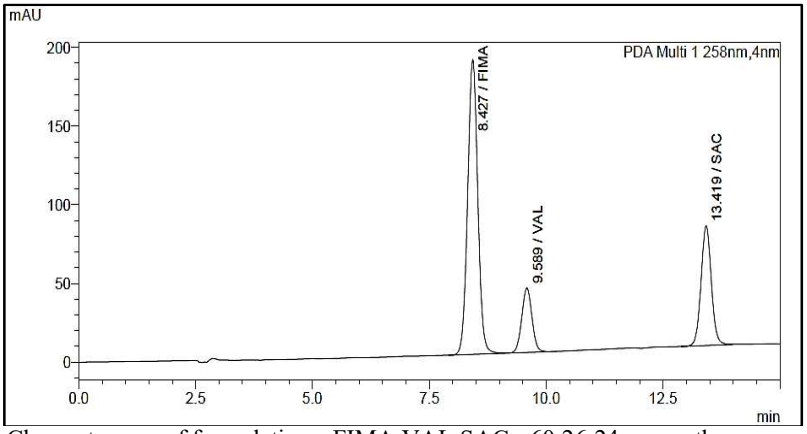


Figure 22: Chromatogram of formulations, FIMA:VAL:SAC - 60:26:24 mg on the proposed method.

Table 6: Assay studies of the prepared formulation by the proposed method.

Drugs	Peak Area (n=6)	Amount of drug estimated	% Assay	% RSD
FIMA	2966209.17	60.394	100.66	0.361
VAL	607663	26.446	100.43	0.239
SAC	1124220.83	24.197	100.20	0.418

6. Conclusion

In the proposed method, the retention time of FIMA, VAL and SAC was found to be 8.5 min, 9.6 min and 13.5 min. The linearity of these drugs were found in the range of 19.2-96 µg/mL for FIMA, 8.32-41.60 µg/mL for VAL and 7.68-38.40 µg/mL for SAC. The regression coefficients (r^2) for FIMA, VAL and SAC were found to be 0.9997, 0.9997 and 0.9996. All the system suitability parameters were within the acceptable limits. The assay of the formulation were performed and obtained within the range of 99-101%. The limit of detection (LOD) for FIMA, VAL and SAC were found to be 3.83 µg/mL, 1.73 µg/mL & 1.81 µg/mL respectively. The limit of quantification (LOQ) for FIMA, VAL & SAC were found to be 11.62 µg/mL, 5.24 µg/mL & 5.49 µg/mL respectively. The recovery studies indicate that the proposed method is accurate. No interfering peaks were found in the chromatogram of the formulation within the run time which indicates excipients used in formulations did not interfere with the estimation of the drugs by the proposed RP-UFLC method.

A RP-UFLC method has been developed for simultaneous estimation of FIMA, VAL and SAC in tablet formulation. The developed method was validated according to ICH Q2 (R1) guidelines and the resolutions between the drugs were sufficient. The low %RSD values of the validation parameters indicate the suitability of the proposed method for routine analysis and quantitative determination of Fimasartan potassium trihydrate, Valsartan and Sacubitril in pharmaceutical formulation by RP-UFLC method. The statistical analysis of data found proves that the method is selective, sensitive, accurate, precise and robust.

7. Acknowledgments

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8. Conflict of Interest

The authors declare no conflict of interest.

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