CHAPTER 4.

SIMULTANEOUS ESTIMATION AND VALIDATION OF SOFOSBUVIR AND VELPATASVIR TABLETS BY RP-HPLC METHOD.

This chapter includes experimental procedures for RP-HPLC method development and its validation for simultaneous estimation of selected double drug dosage form SOFOSVEL tablet.

Sofusbuvir and Velpatasvir are represented as SOF and VEL respectively in this chapter.

4.1 Sofosvel tablet.

SOFOSVEL tablet has a composition of SOF-400mg and VEL-100mg. Sofosvel [72-74] is orally taken for the treatment of adult patients with chronic hepatitis C virus (HCV). The inactive ingredients include cellulose ,Copovidone, Magnesium stearate and Croscarmellose sodium. Red Iron oxide, polyethylene glycol, polyvinyl alcohol are incorporated in the tablet film.

4.1 A Molecular structure of SOF.

Structure of SOF is shown in the Fig 4.1

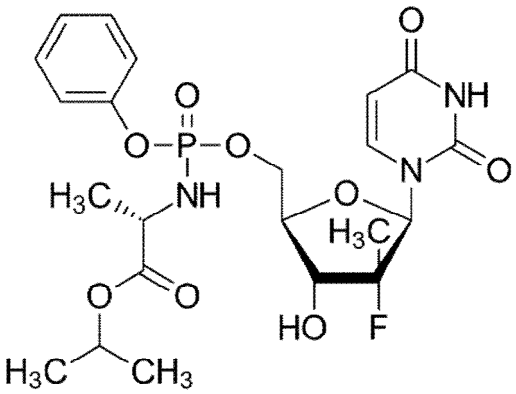


Fig 4.1 Structure of SOF

4.1 B. Properties of SOF

Table 4.1 Properties of SOF

IUPAC Name	(S)-Isopropyl-2-((S)-(((2R, 3R, 4R, 5R)-5-(2, 4-dioxo-3, 4-dihydropyrimidin-1 (2H)-yl)-4-fluoro-3-hydroxyl-4-methyltetrahydrofuran-2-yl) methoxy)-(phenoxy) phosphorylamino) propanoate
Molecular formula	$C_{22}H_{29}FN_3O_9P$
Molecular weight	529 g/mol
CAS No.	1190307-88-0
Description	White to off-white crystalline solid
Solubility	Soluble in Water, Methanol , Acetonitrile, Acetone ,
Melting point	120˚C
pKa	9.3
Category	Anti-Viral

4.1 C Pharmacodynamics of SOF

SOF is a Hepatitis C virus NS5B polymerase nucleotide inhibitor and it stops the HCV RNA replication. In the liver cells SOF is transformed in to active Uridine triphosphate form which acts against the NS5B polymerase.

4.1 D Pharmacokinetics of SOF.

SOF exhibited good stability in gastric and intestinal fluids. It is well absorbed and highly extracted by the liver .SOF is not accumulated after repeated dosing.

4.2. Velpatasvir.

4.2 A Molecular structure of VEL.

Structure of VEL is shown in the Fig 4.2.

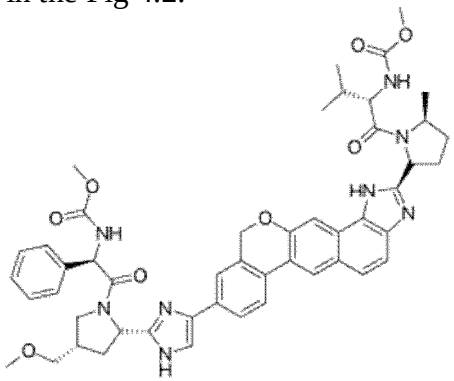


Fig 4.2 Structure of VEL

4.2 B .Properties of VEL

TABLE 4.2 Properties of VEL

IUPAC Name	Methyl ((R)-2-((2S,4S)-2-(5-(2-((2S,5S)-1-((methoxycarbonyl)-L-valyl)-5-methylpyrrolidin-2-yl)-1,11-dihydroisochromeno[4',3':6,7]naphtho[1,2-d]imidazol-9-yl)-1H-imidazol-2-yl)-4-(methoxymethyl)pyrrolidin-1-yl)-2-oxo-1-phenylethyl)carbamate
Molecular formula	$C_{49}H_{54}N_8O_8$
Molecular weight	883 g/mol
CAS No	1377049-84-7
Physical state	White or off white solid
Solubility	Soluble in Acetone, Acetonitrile, Methanol and water.
Melting point	170˚C
pKa	3.2 and 4.6
Category	Anti-Viral

4.2C Pharmacodynamics of VEL.

VEL shows its antiviral property by preventing the HCV nonstructural protein NS5A from replicating and also from HCV virion arrangement. The combined dose of SOF and VEL show significant antiviral properties due to their combined antiviral effect and absence of cross resistance between SOF and VEL.

4.2 D Pharmacokinetics of VEL.

VEL shows good plasma protein binding capacity. After oral administration the drug is quickly distributed to the tissues majority of it is absorbed by the liver.

EXPERIMENTAL PROCEDURE.

4.3 Instrumentation.

List of instruments used in the experiment are mentioned in the Table 3.2. List of HPLC grade chemicals used in the present experiment are mentioned in the Table 3.3.

4.4 Method development.

4.4.1. Selection of wavelength.

Clear solutions of SOF and VEL were prepared in Methanol and scanned with the UV-Vis spectrometer in the entire UV region. The maximum combined UV absorption response for both the drugs was observed at 254 nm of wavelength. This is shown in the Fig 4.3.

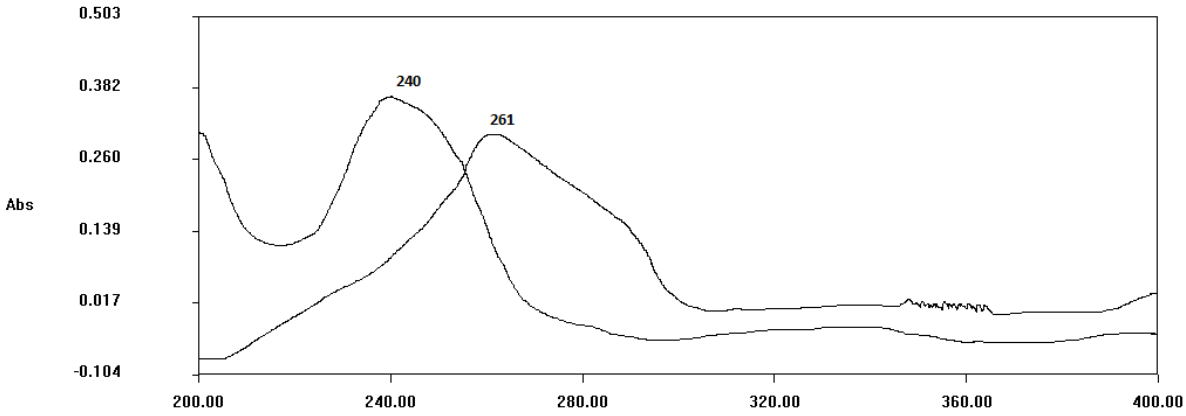


Fig 4.3 Overlain spectrum of SOF and VEL.

4.4.2 Experimentation for the optimized chromatographic conditions.

The basic principle involved in the selection of chromatographic conditions is described in the section 3.1.3 c. A 1000 µg/mL HPLC grade combined solution of SOF and VEL was prepared to run the trial chromatograms.

TRIAL 1

Column : INERTSIL C-18 (250mm × 4.6 mm, 5 µm)

Mobile phase: Degassed Acetonitrile

Diluent : Acetonitrile

Detector wavelength – 254 nm

Column temperature – 25 º C

Injection volume – 20µL

Flow rate 1.0mL/ min

Run time 10 minutes

Elution Type – Isocratic mode.

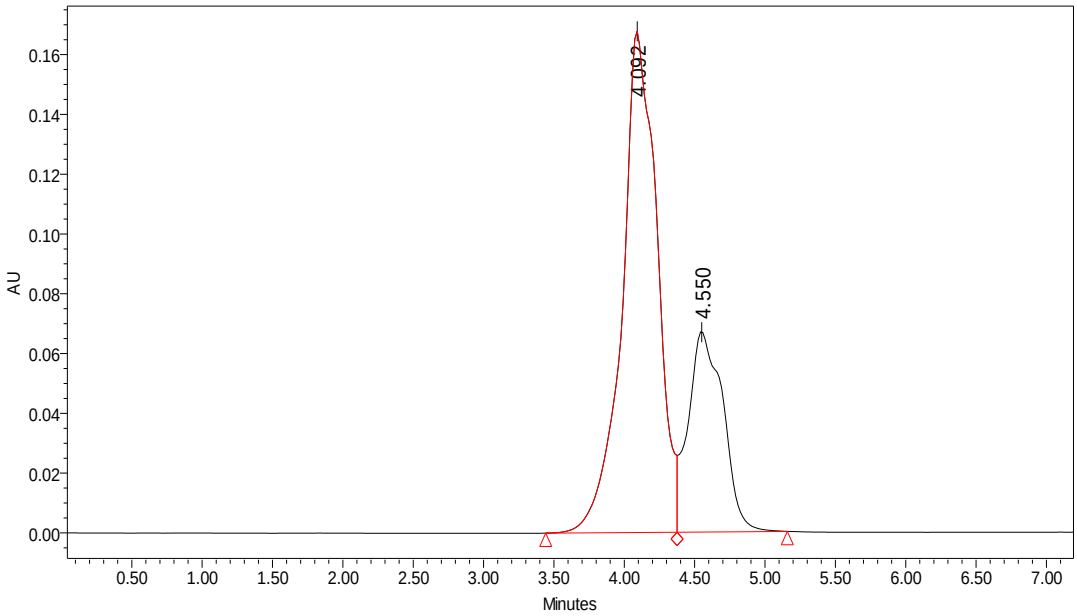


Fig 4.4 Chromatogram of SOF and VEL. (Trial 1)

Observation: RT of SOF was 4.092 minutes and VEL was 4.55 minutes.

Two peaks were merged and not well separated. Hence a new trial with a different mobile phase was carried again.

TRIAL 2

Column: INERTSIL C-18 (250mm × 4.6 mm, 5 µm)

Mobile phase: Degassed Acetonitrile and Methanol in the ratio (90:10) v/v

Diluent: Acetonitrile

Detector wavelength – 254 nm

Column temperature – 25 º C

Injection volume – 20µL

Flow rate - 1.0mL/ min

Run time – 10 minutes

Elution Type – Isocratic mode.

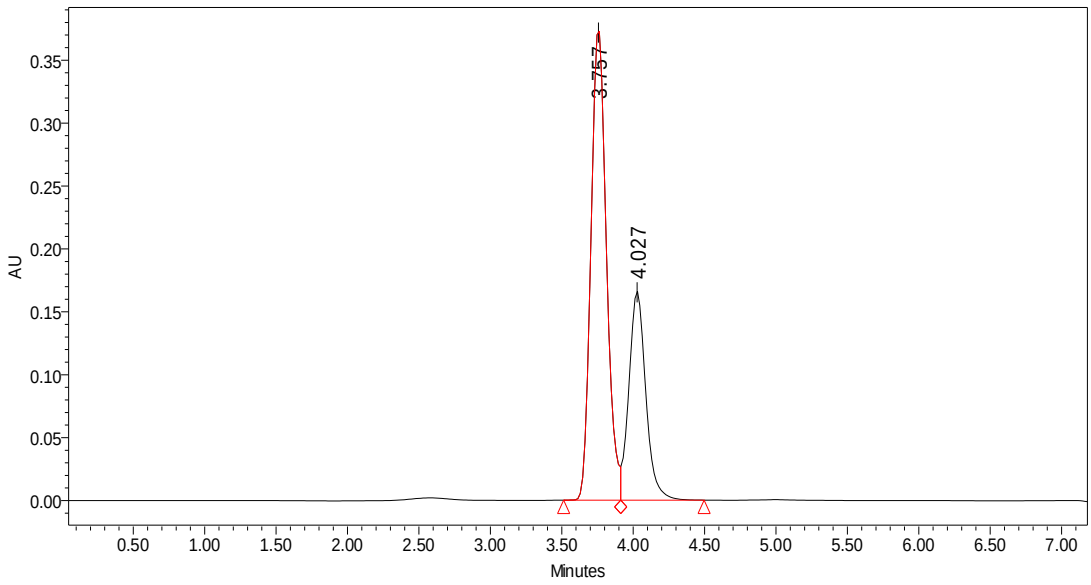


Fig 4.5 Chromatogram of SOF and VEL (Trial 2)

Observation : RT of SOF was 3.757 minutes and VEL was 4..027 minutes. The peaks were overlapping and not well resolved. Hence another trial was carried.

TRIAL 3.

Column : INERTSIL C-18 (250mm × 4.6 mm, 5 µm)

Mobile phase : Degassed Acetonitrile and Methanol in the ratio (80:20) v/v

Diluent :Acetonitrile

Detector wavelength – 254 nm

Column temperature – 25 º C

Injection volume – 20µL

Flow rate 1.0mL/ min

Run time 10 minutes

Elution Type – Isocratic mode

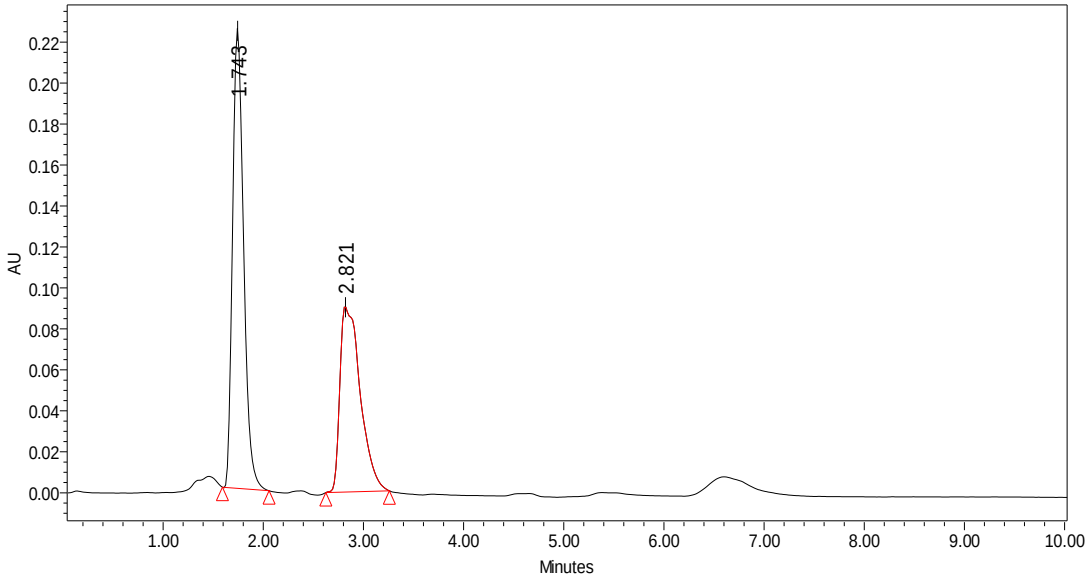


Fig 4.6 Chromatogram of SOF and VEL (Trial 3)

Observation: RT for SOF was 1.747 minutes and 2.821 minutes for VEL.

The baseline was not straight. The system evaluation parameters were not within the acceptance region. Hence trial 4 was carried.

TRIAL 4.

Column : INERTSIL C-18 (250mm × 4.6 mm, 5 µm)

Mobile phase: Methanol and phosphate buffer in the ratio (60:40) v/v

Diluent: Methanol.

Detector wavelength – 254 nm

Column temperature – 25 º C

Injection volume – 20µL

Flow rate - 1.0mL/ min

Run time – 8 minutes

Elution Type – Isocratic mode.

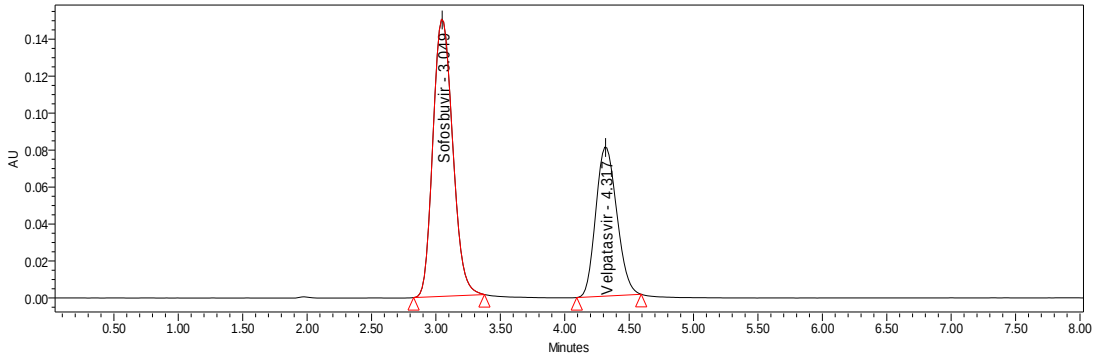


Fig 4.7 Chromatogram of SOF and VEL (Trial 4)

Observation: The RT of SOF was 3.049 minutes and that of VEL was 4.317 minutes. The two peaks were well resolved. The system evaluation parameters were all within the acceptance range. Hence the conditions of Trial 4 were selected as the optimized chromatographic conditions.

4.4.3 Preparation of buffer solution.

Phosphate buffer solution of 0.05M was prepared by combining 6.67 grams of Potassium dihydrogen phosphate and 8.55 grams of Di potassiumhydrogen phosphate in a one liter flask. To this 800ml of HPLC grade water was added, sonicated thoroughly and the final volume was adjusted to one liter mark in the flask . The final

pH of the solution was maintained at 3.2 with careful addition of Orthophosphoric acid. The buffer solution was micro filtered before using in the HPLC analysis.

4.4.4. Preparation of mobile phase.

Mobile phase was composed of a mixture of HPLC grade Methanol and phosphate buffer mixed in the ratio of 60:40 v/v. Mobile phase was also used as the diluent in this analysis.

4.4.5. Preparation of SOF and VEL combined standard API solution.

SOF Stock solution: 100mg of SOF pure API sample was dissolved and diluted with the mobile phase in a 100mL volumetric flask up to the mark to get a solution of 1000 µg/mL.

VEL stock solution: 100 mg of VEL pure API sample was dissolved and diluted with the mobile phase in a 100mL volumetric flask up to the mark to get a solution of 1000 µg/mL

Working standard Solution of SOF and VEL combined was prepared by adding 4mL of SOF and 1mL of VEL from their respective stock solution into a single 100mL volumetric flask and diluted up to the mark and the solution of this flask was a combined solution with a concentration of 40µg/mL SOF and 10µg/mL VEL.

4.4.6. Preparation of SOF and VEL combined tablet sample solution.

Ten Sofosvel tablets having a dosage of SOF 400mg and VEL 100mg were weighed and average weight of single tablet noted. These tablets were powdered and weight of powder equal to one tablet weight was introduced into a single 100mL flask, dissolved and diluted up to the mark with mobile phase.

One mL of the above solution was further diluted in a 100mL flask to get a concentration of 40 µg/mL SOF and 10 µg/mL VEL combined test sample solution.

4.4.7. Preparation of Placebo Solution of Sofosvel Tablet.

The inactive ingredients in the Sofosvel tablet as listed in section 4.1 were collected and a clear HPLC grade solution was prepared to record the placebo chromatogram.

4.4.8 Blank solution: The mobile phase was used as the blank solution.

4.4.9. Optimized Chromtograms.

The procedure followed to record the optimized chromatograms of the standard and the tablet sample was the same as mentioned in the section 3.2.7

Optimized chromatograms of the standard and the tablet sample are shown in the Fig 4.8 and 4.9.

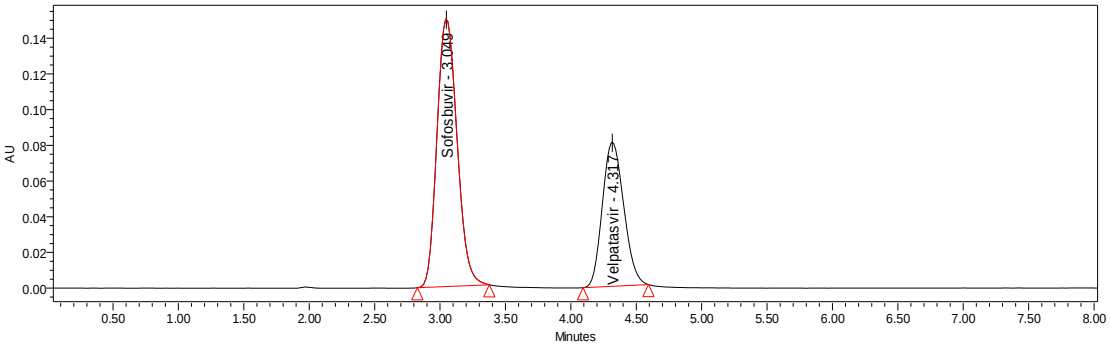


Fig 4.8 Standard chromatogram of SOF and VEL

Table 4.3 System evaluation parameters of the Standard API chromatogram

Drug	RT (min)	Peak area(AU)	Theoretical plates	Resolution	Tailing factor.
SOF	3.049	9431581	11036	---	1.18
VEL	4.317	323915	8372	4.29	1.28

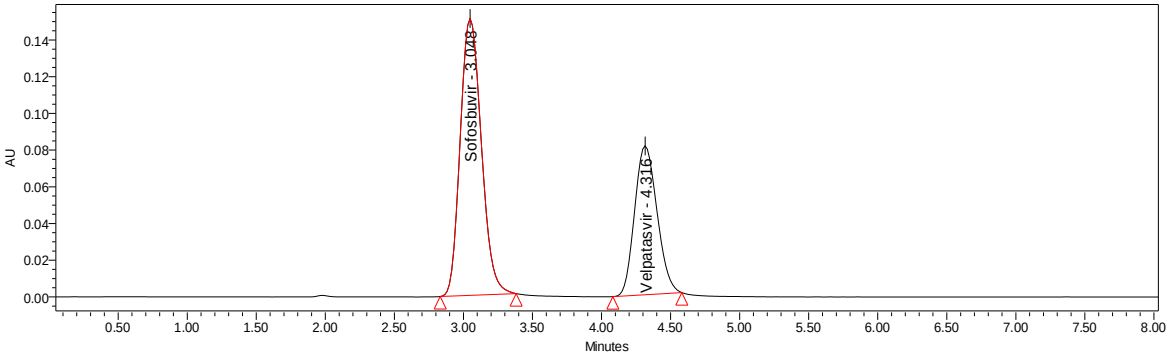


Fig 4.9 Tablet sample chromatogram of SOF and VEL

Table 4.4 System evaluation parameters of the Tablet sample chromatogram.

Drug	RT(min)	Peak area (AU)	Theoretical plates	Resolution	Tailing factor.
SOF	3.048	9438247	11023	---	1.14
VEL	4.316	323209	8325	4.34	1.128

Result: All the system evaluation parameters for the optimized chromatograms of combined solution of SOF and VEL in its pure form and the tablet dosage form were within the acceptance range.

4.4.10 METHOD VALIDATION.

A. **System Suitability** The experimental procedure for checking the system suitability were the same as mentioned in the section 3.1.10 a.

System suitability chromatograms are shown in the figure from 4.10 (a to e)

Observations from the chromatograms are presented in the table 4.5 a and 4.5 b for SOF and VEL.

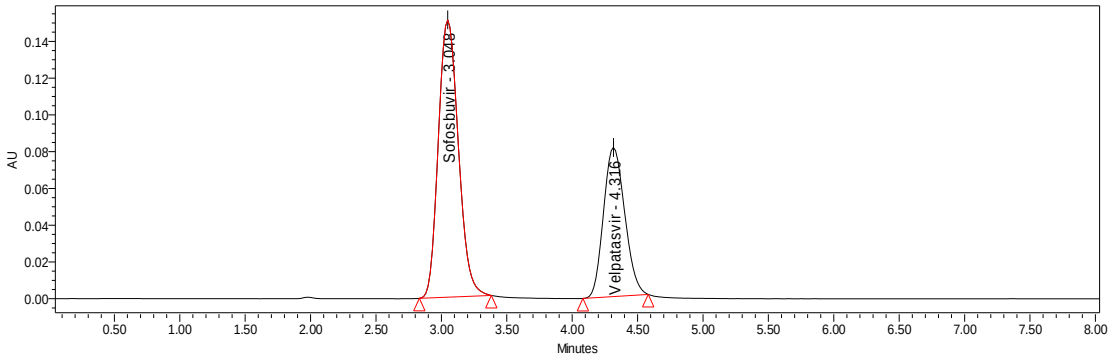


FIG 4.10 a System suitability chromatogram Trial 1

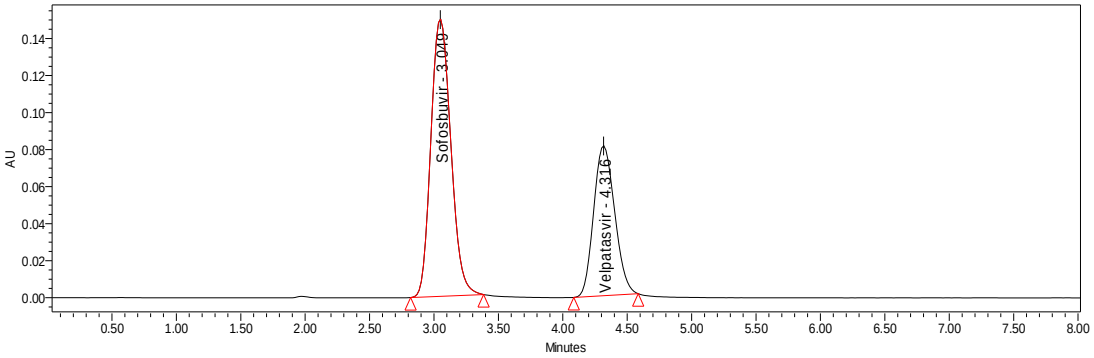


Fig 4.10 b System suitability chromatogram Trial 2

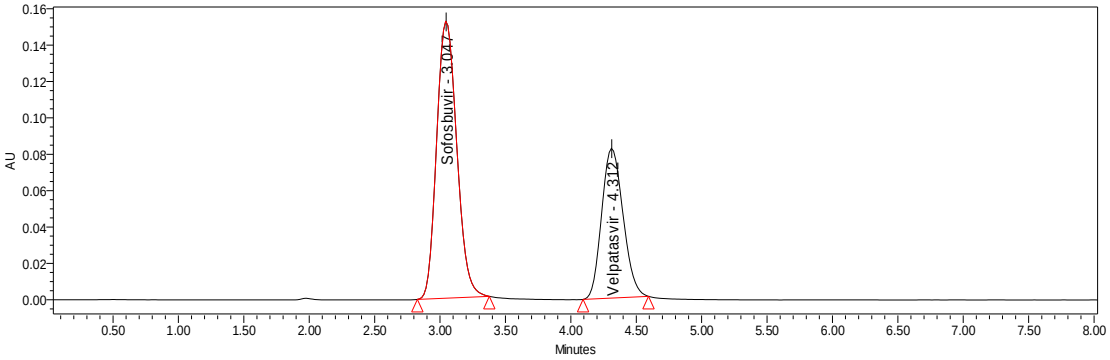
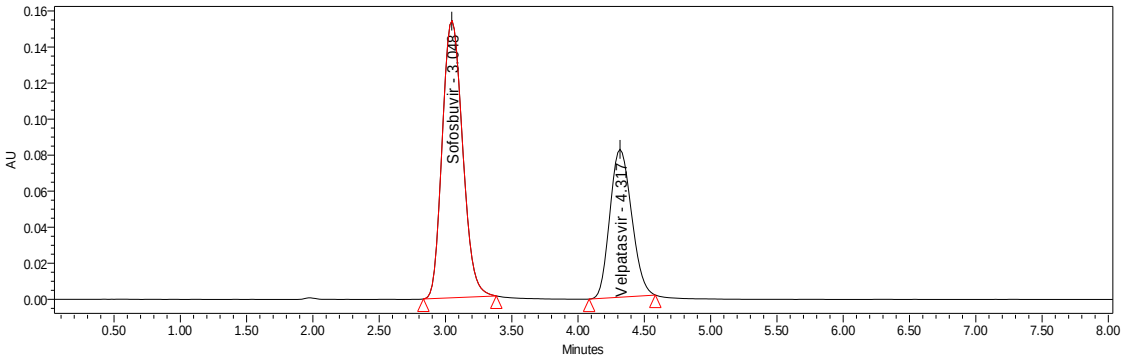


Fig 4.10 c System suitability chromatogram Trial 3



4.10 d System suitability chromatogram Trial 4

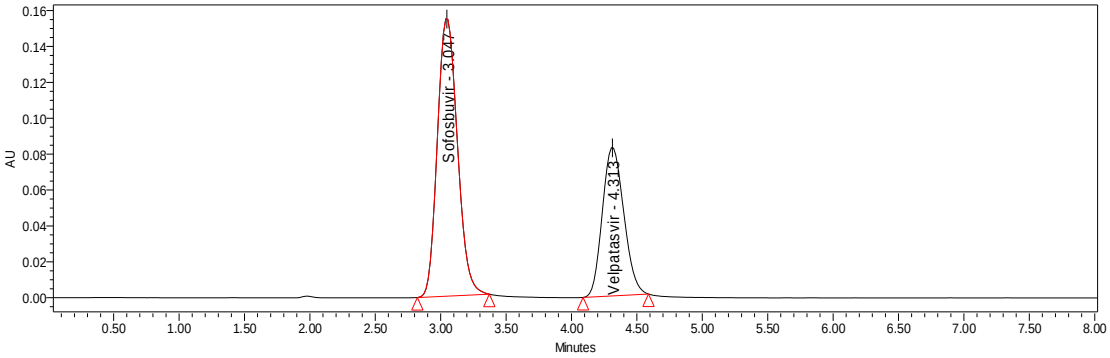


Fig 4.10 e System suitability chromatogram Trial 5

Table 4.5 a System Suitability Parameter for SOF

Trial	RT(min)	Peak Area(AU)	Theoretical plates	Tailing factor
1	3.048	9438247	11023.845712	1.14721
2	3.049	9436021	11010.547812	1.13384
3	3.047	9431581	11036.874214	1.18742
4	3.048	9432036	11027.254178	1.16547
5	3.047	9433819	11084.658952	1.17485
Mean	3.0478	9434755	11036.825471	1.16
SD	0.000837	3358.178	28.41	0.0209
% RSD	0.027451	0.270438	0.26	1.81

Table 4.5 b System Suitability Parameter of VEL

Trial	RT(min)	Peak Area(AU)	Theoretical plates	Tailing factor	Resolution
1	4.316	323209	8325.874512	1.284572	4.34
2	4.316	323181	8384.547862	1.254872	4.29
3	4.312	323028	8314.875424	1.278451	4.31
4	4.317	323915	8372.784518	1.287451	4.30
5	4.313	324059	8392.084512	1.298745	4.34
Mean	4.3148	3238476	8357.4	1.274	4.315
SD	0.002168	1588.8	35.5358	0.015	0.0207
% RSD	0.050244	0.289823	0.43	1.19	0.48

B. Specificity.

The API sample and the tablet sample of combined solution of SOF and VEL at 40µg/mL and 10µg/mL respectively were injected in to the HPLC system to record their chromatograms. The chromatograms of the blank and the placebo were also recorded.

The standard API chromatogram is shown in the Fig 4.8.The tablet sample chromatogram is depicted in the Fig 4.9.

The blank chromatogram is depicted in the Fig 4.11

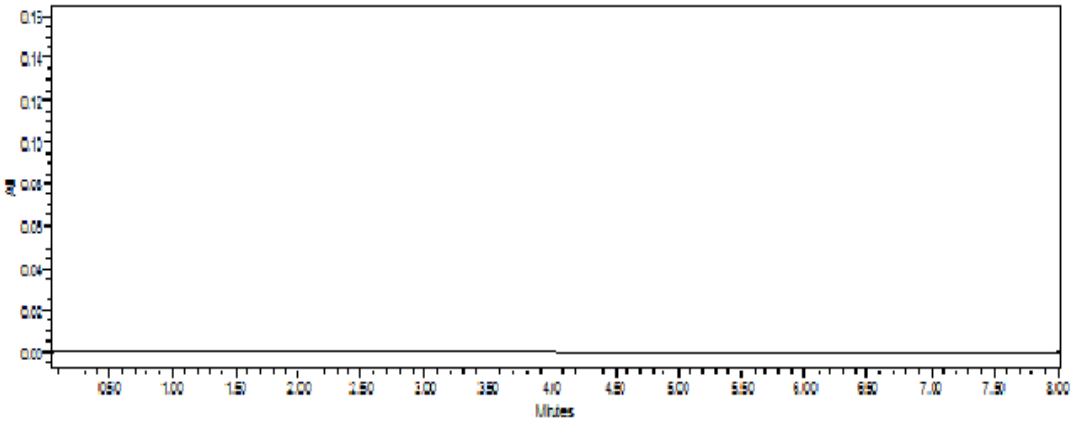


Fig 4.11 Optimized Blank chromatogram

The placebo chromatogram is shown in the Fig 4.12

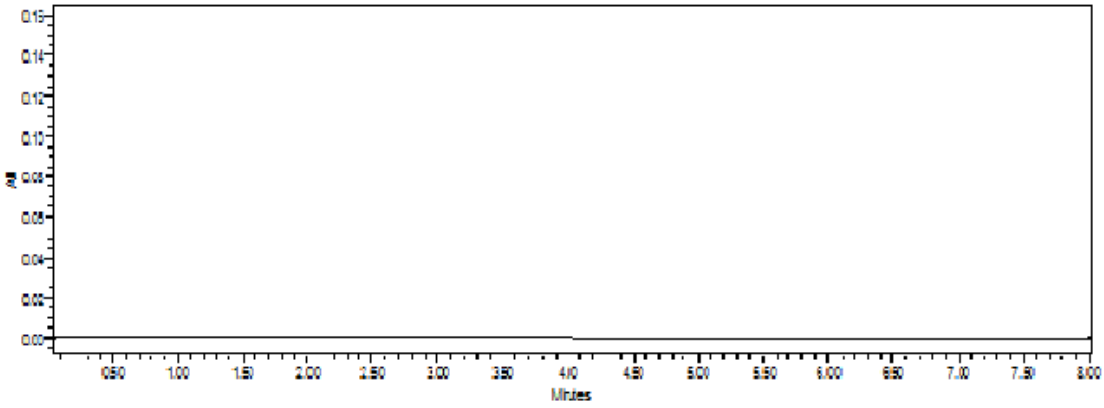


Fig 4.12 Optimized Placebo Chromatogram

The peak characteristics of the standard API sample and the tablet sample are shown in the Table 4.3 and 4.4.

Result: The blank and the placebo chromatograms has shown no peak at the RT of neither at SOF nor at the RT of VEL.

The API and the sample chromatograms showed peaks at identical RT that is 3.04 minutes for SOF and 4.31 minutes for VEL. There was no interference due to the excipients present in the placebo at the RT of the drugs in the sample chromatogram. Hence the developed method was valid with respect to Specificity parameter.

C. Linearity.

Preparation of Working standard solution.

The procedure for the preparation of the working standard solutions of SOF and VEL in both pure API form and Tablet form is explained in the section 4.4.5 and 4.4.6.From the working standard solutions linearity level solutions of SOF ranging from 20 µg/mL to 80µg/mL and of VEL ranging from 5 µg/mL to 20 µg/mL in combination with each other at equal intervals are prepared in seven different 10 mL flask . Each of the combination linearity level solution is injected in to the HPLC system thrice and the chromatograms were recorded. These are shown in the Fig 4.13 (a to e)

A Linear regression graphs were plotted between peak area (y axis)of the chromatograms and their respective linearity range concentration (x axis) separately for SOF and VEL. The graphs are shown in the Fig 4.14 a and b.

The linearity chromatograms values are depicted in the Table 4.6.

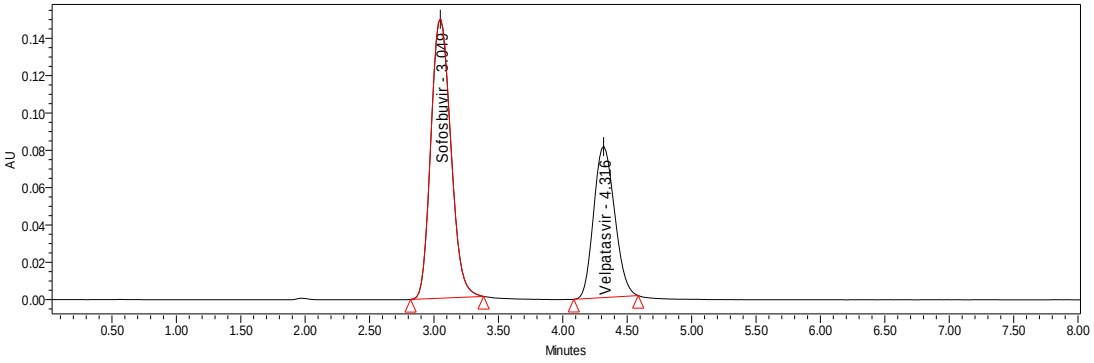


Fig 4.13 a Linearity chromatogram Level 1.

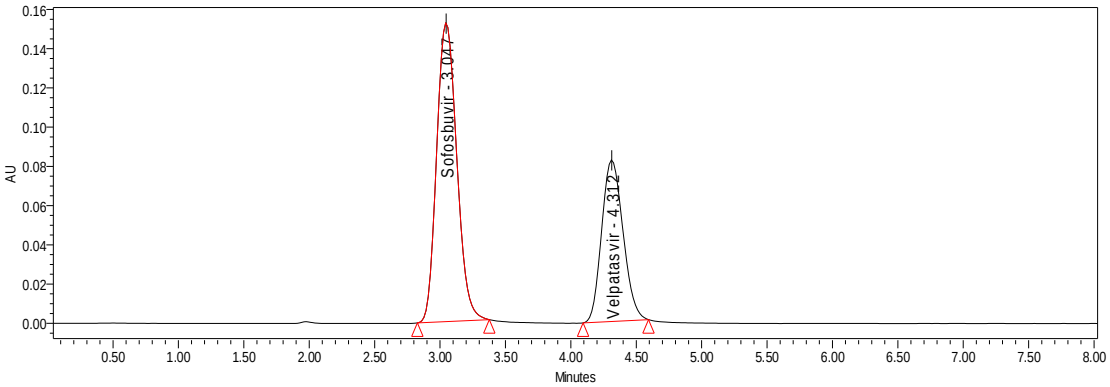


Fig 4.13 b Linearity chromatogram Level 2

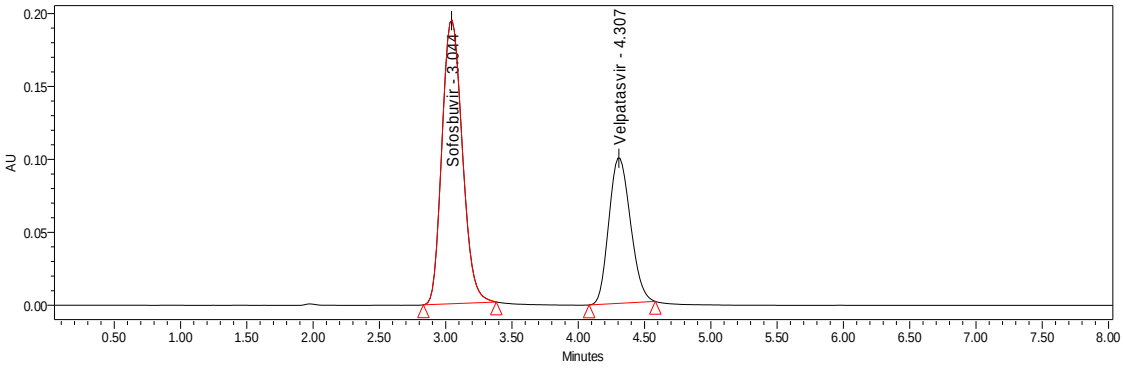


Fig 4.13 c Linearity chromatogram Level 3

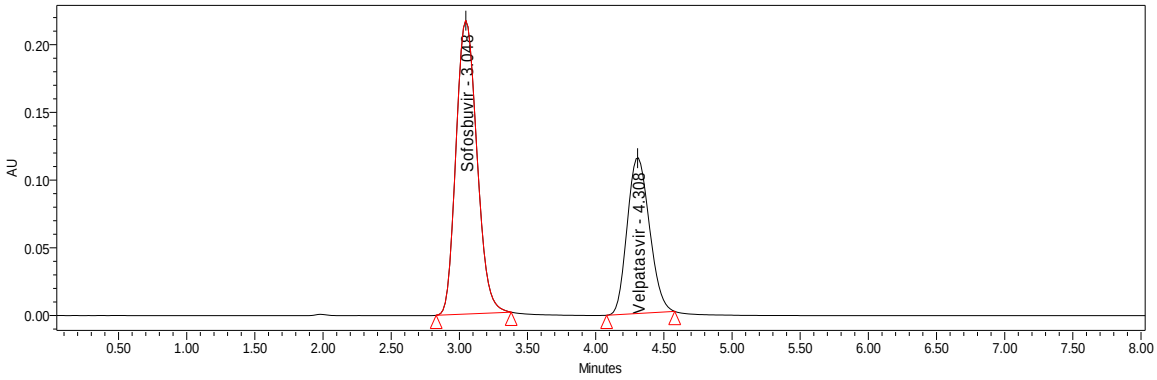


Fig 4.13 d Linearity chromatogram Level 4.

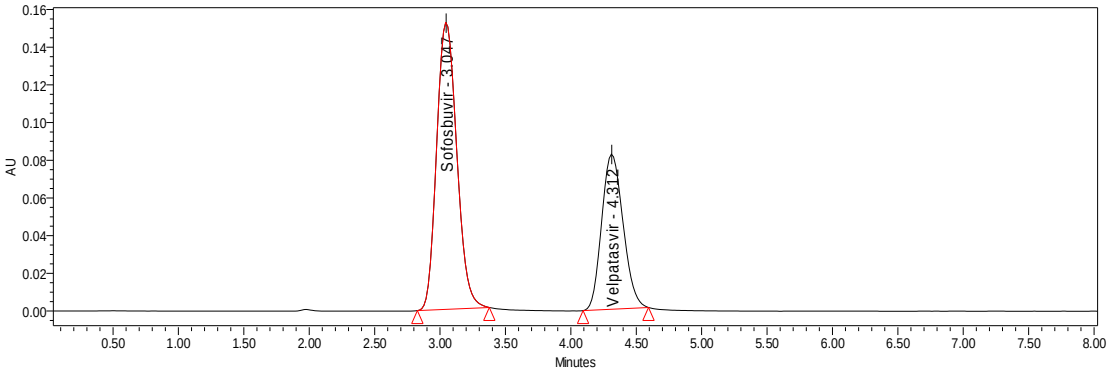


Fig 4.13 e Linearity chromatogram Level 5

Table 4.6 Linearity Parameter

Linearity level	Concentration (µg/mL)	Average Peak area (AU) (SOF)	Concentration (µg/mL)	Average Peak area (AU) (VEL)
1	20	4719376	5	161774
2	30	7079064	7.5	242661
3	40	9438751	10	323547
4	50	11798439	12.5	404434
5	60	14158127	15	485321
6	70	16517815	17.5	566208
7	80	18477503	20	637095

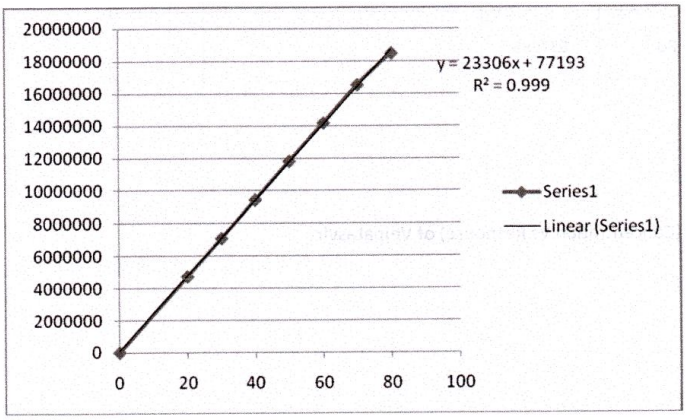


Fig 4.14 a Linearity graph of SOF

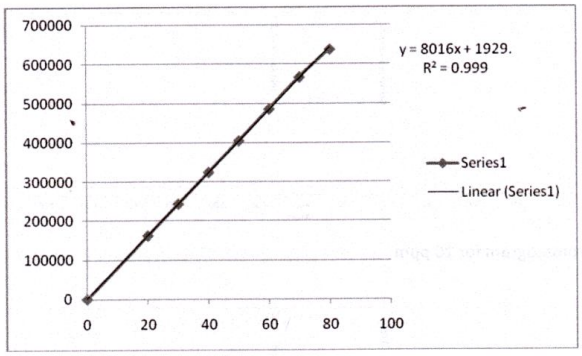


Fig 4.14 b Linearity graph of VEL

Result: The linear regression line and the correlation coefficient (R^2) value established a linear relationship between the peak area and the concentration of the solution. The linearity parameter is validated.

D. Accuracy.

The principle and the experimental procedure for the accuracy determination is mentioned in the section 3.1.10.

Preparation of Accuracy level solutions.

A mixture 20µg/mL of SOF and 5µg/mL of VEL was taken for the preparation of 50% accuracy level solution. A mixture 40µg/mL of SOF and 10µg/mL of VEL was taken for the preparation of 100% accuracy level solution. A mixture 60µg/mL of SOF and 15µg/mL of VEL was taken for the preparation of 150% accuracy level solution.

These accuracy level solutions were prepared in a 100 mL volumetric flask from the 1000 µg/mL stock solution by suitable dilution as mentioned in the section 4.4.5 and 4.4.6. To these solutions tablet sample solution of fixed concentration at 40 µg/mL 0f SOF and 10 µg/mL of VEL was added. Mixed well, sonicated thoroughly and micro filtered before injecting in to the sample port of the HPLC system.

The Accuracy chromatograms are depicted in the Fig 4.15 (a to c)

The observations from the accuracy parameter studies are depicted in the Table 4.7.

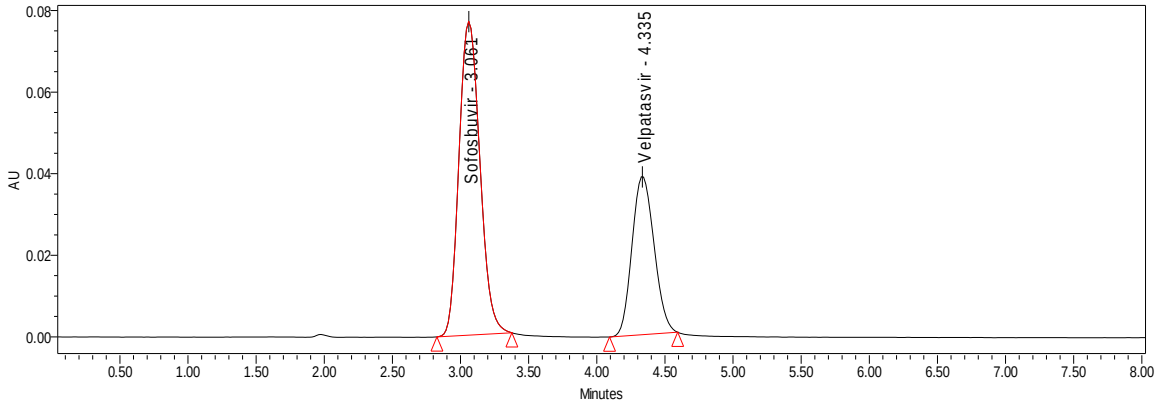


Fig 4.15 a Accuracy chromatogram at 50% concentration.

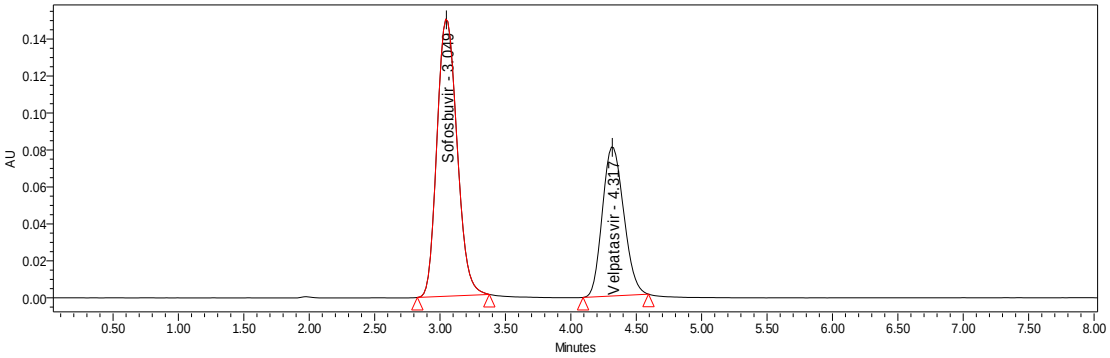


Fig 4.15 b Accuracy chromatogram at 100% concentration.

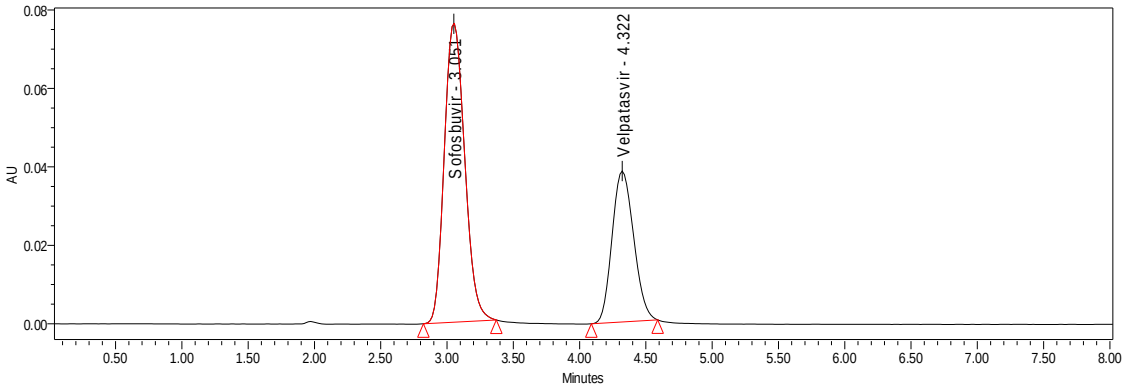


Fig 4.15 c Accuracy chromatogram at 150% concentration.

Table-4.7 Accuracy parameter

Spike level concentration		SOF			VEL		
%	Trial number	Amount added µg/mL	Amount Recovered µg/mL	% Recovery	Amoun added µg/mL	Amount Recovered (µg/mL)	% Recovery
50	1	20	20.01	100.05	20	20.45	102.25
	2	20	19.91	99.5	20	19.84	99.2
	3	20	20.08	100.4	20	20.07	100.35
100	1	40	40.03	100.07	40	39.45	98.62
	2	40	39.98	99.95	40	40.07	100.17
	3	40	39.91	99.77	40	39.93	99.82
150	1	60	60.02	100.03	60	60.02	100.03
	2	60	60.07	100.11	60	59.98	99.96
	3	60	60.04	100.06	60	60.07	100.11
			Mean	99.993			100.05
			%RSD	0.68			0.327

RESULT . The percent recovery of the drug at 50 %, 100% and 150% concentration level were found to be with in the acceptance range as per the guidelines. Hence the developed method was accurate.

E. PRECISION

All the HPLC runs were carried out with working standard combined SOF and VEL standard API solution for system precision studies and with working standard combined solution of Sofosvel tablet sample for method precision studies.

The preparation of the working standard solution is explained in the section 4.4.5 and 4.4.6.

The standard and the sample solution were injected in to the system in six replicates and the peak area were noted for all the chromatograms. The %RSD values for the peak area were calculated.

The chromatograms for system precision are given in the Fig from 4.16 (a to e).

The values of system precision for the combined sample are depicted in the Table 4.8.

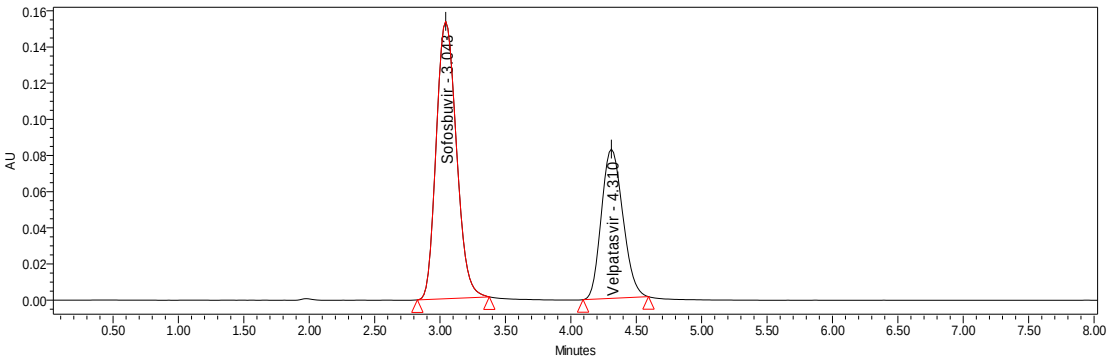


Fig 4.16 a Chromatogram for System precision (Trial 1)

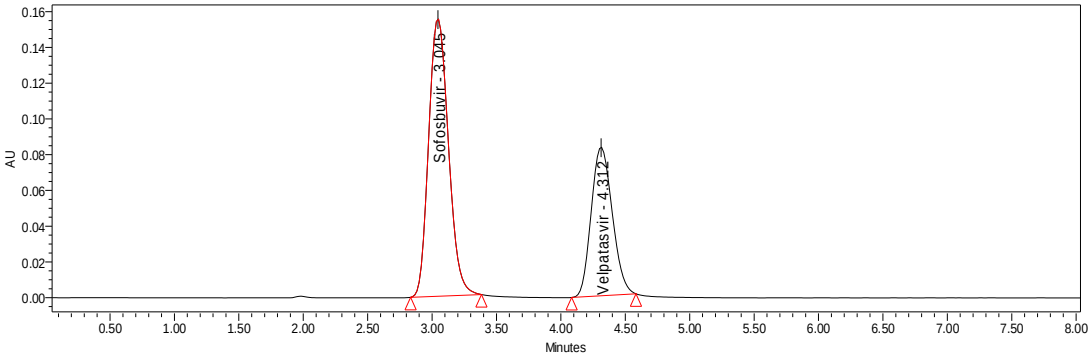


Fig 4.16 b Chromatogram for System precision (Trial 2)

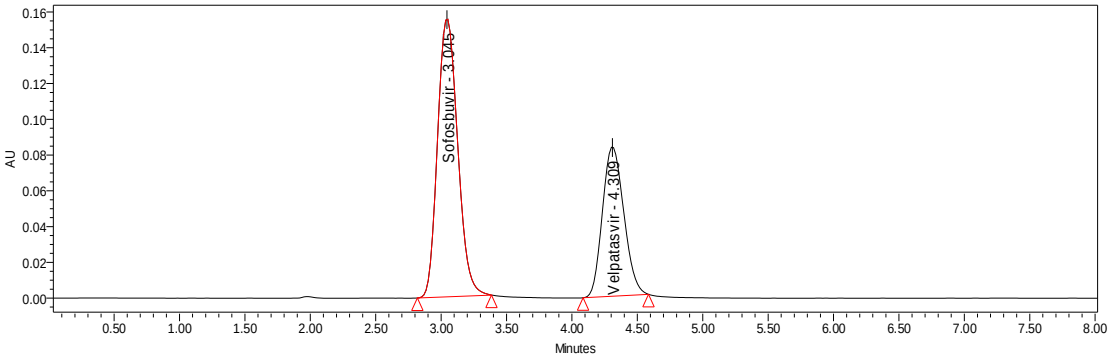


Fig 4.16 c Chromatogram for System precision (Trial 3)

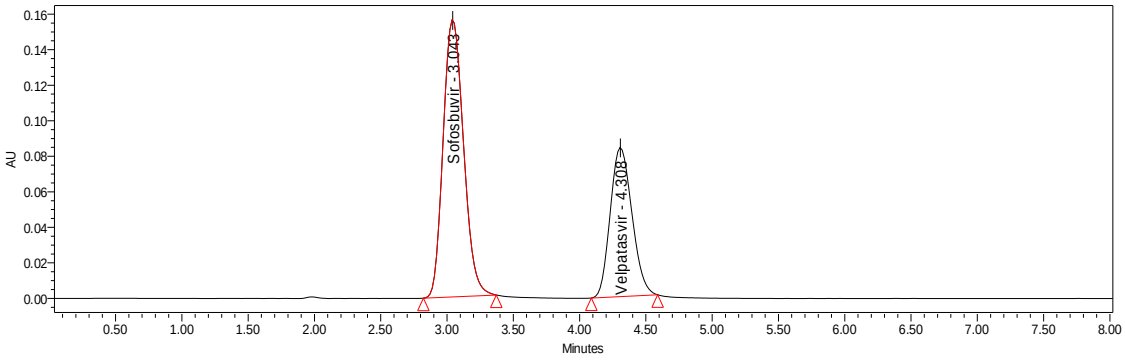


Fig 4.16 d Chromatogram for System precision (Trial 4)

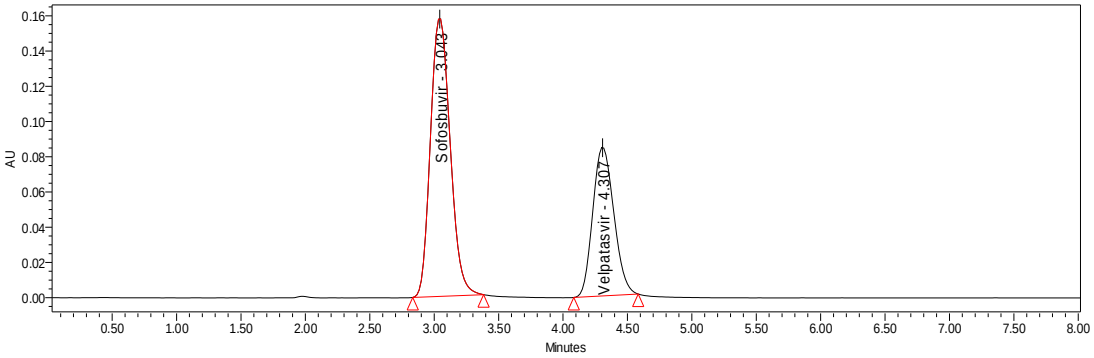


Fig 4.16 e Chromatogram for System precision (Trial 5)

Table 4.8 System precision parameter.

Trial	SOF		VEL	
	RT (min)	Peak area(AU)	RT (min)	Peak area(AU)
1	3.043	9437784	4.310	323112
2	3.045	9437412	4.312	323452
3	3.045	9430257	4.309	323742
4	3.043	9438431	4.308	323047
5	3.043	9438754	4.307	323087
Mean	3.0438	94367079	4.3092	3231472
SD	0.001	10475.12	0.0019	7452.4712
% RSD	0.04	0.7842	0.04	0.752411

The chromatograms for method precision are depicted in the Fig from 4.17 (a to f) and the values are given in the Table 4.9.

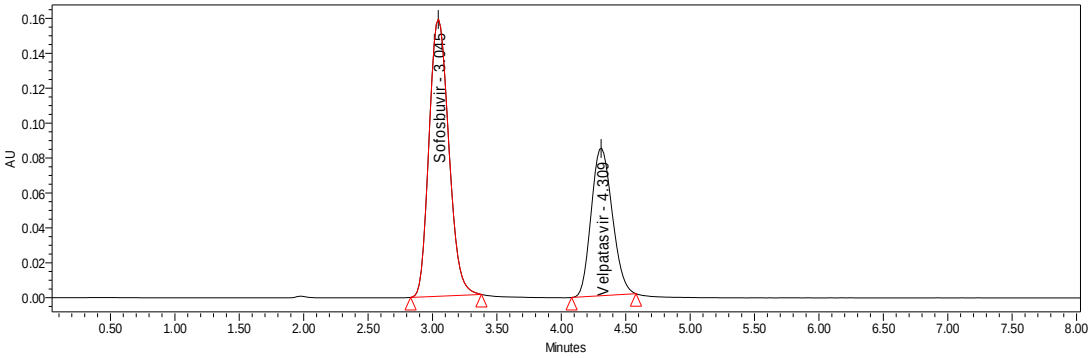


Fig 4.17 a Method precision chromatogram Trial 1

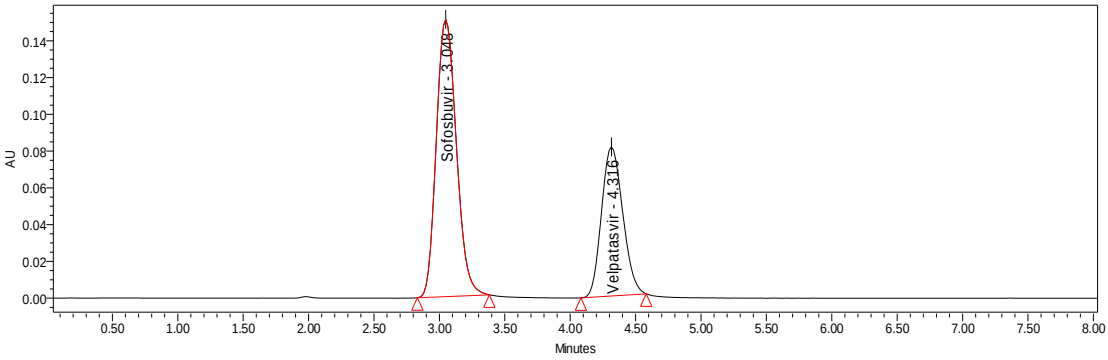


Fig 4.17 b Method precision chromatogram Trial 2

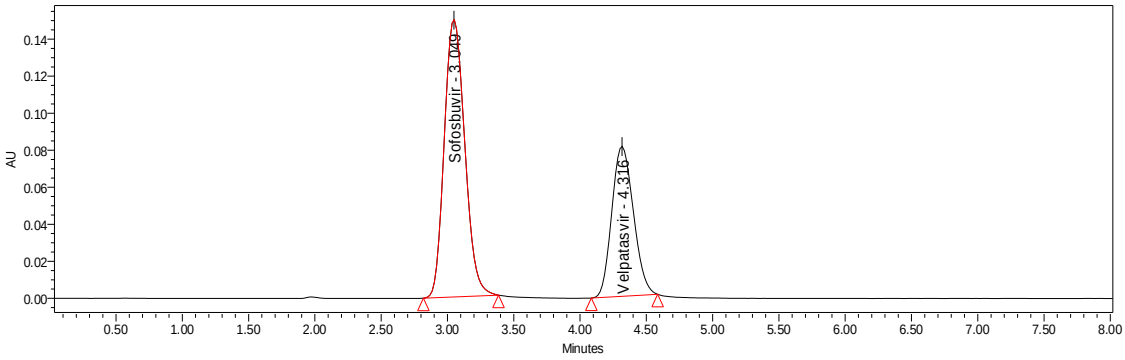


Fig 4.17 c Method precision chromatogram Trial 3

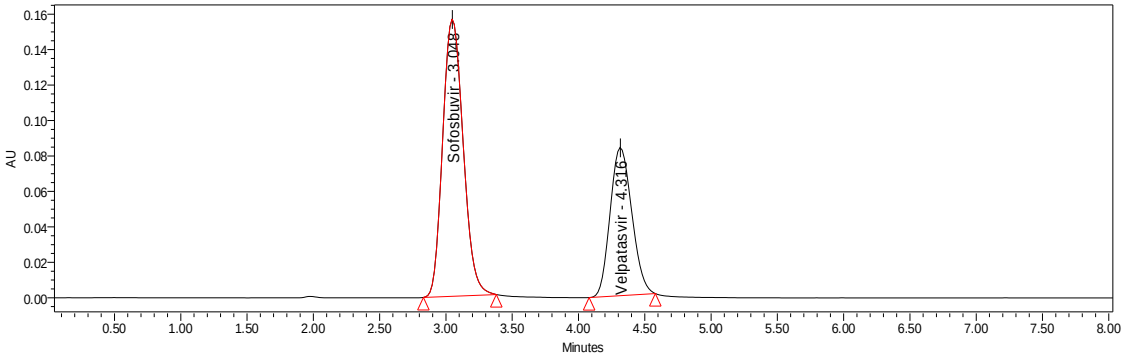


Fig 4.17 d Method precision chromatogram Trial 4

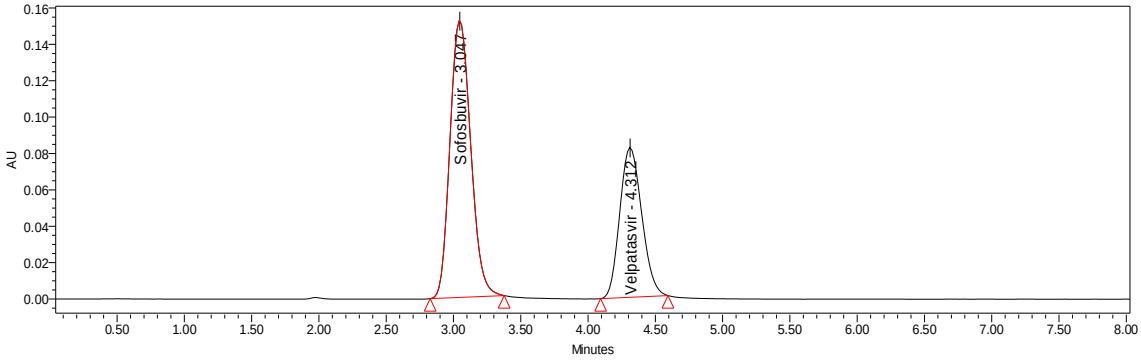


Fig 4.17 e Method precision chromatogram Trial 5

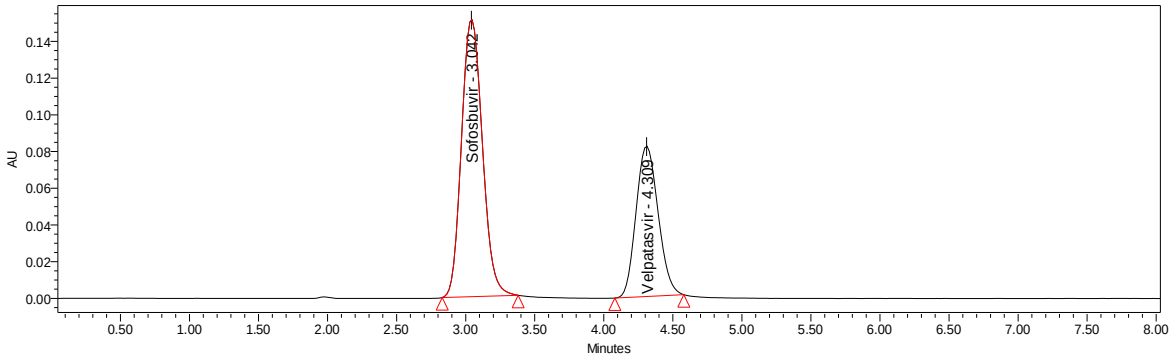


Fig 4.17 f Method precision chromatogram Trial 6

Table 4.9 Method precision parameter

Trial	SOF		VEL	
	Peak area (AU)	**% Assay**	**Peak area (AU)**	**% Assay**
1	9432571	99.25	323584	99.54
2	9438475	99.12	323054	99.72
3	9434752	98.12	323847	99.31
4	9430487	99.52	323751	99.84
5	9436547	98.84	323814	99.42
6	9437841	99.54	323745	99.32
Mean	9438845	99.56	323875	99.87
SD	147205	0.54213	3240.5412	0.7845
% RSD	0.7451	0.412	0.54721	0.874654

RESULT: The % RSD values for all the five chromatograms of the system precision and the six chromatograms of method precision were within the acceptance limit that is less than 2. The assay percent calculated from the Method precision chromatograms were also within acceptance range that is in between 98% to 102%. All the values were in close agreement and hence the developed method was precise.

F. LOD and G.LOQ

The experimental procedure was the same as mentioned in the section 3.1.10.The values are incorporated in the Table 4.10

Table 4.10 Linearity regression line values.

Drug	**Slope**	**Intercept**	**Standard deviation of the intercept**	**Regression Coefficient (R^2)**	**LOD (µg/mL)**	**LOQ (µg/mL)**
SOF	23306	77193	3358.17	0.999	0.475	1.44
VEL	8016	1929	1588.8	0.999	0.65	1.98

H. Robustness.

The basic principle, the experimental procedure and the experimental conditions maintained to validate the Robustness parameter in the present simultaneous work were also the same as mentioned in the section 3.1.10 H 1, 2, 3.

The chromatograms recorded in the Robustness experiment are shown in the Fig 4.18 to 4.20 (a and b). The values of Robustness parameter are presented in the Table 4.11.

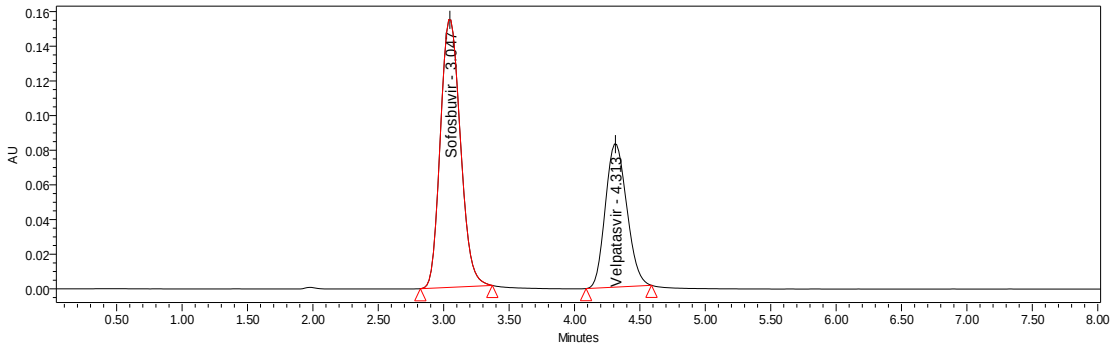


Fig 4.18 a Robustness Chromatogram (Mobile Phase Flow rate at 0.8mL/min)

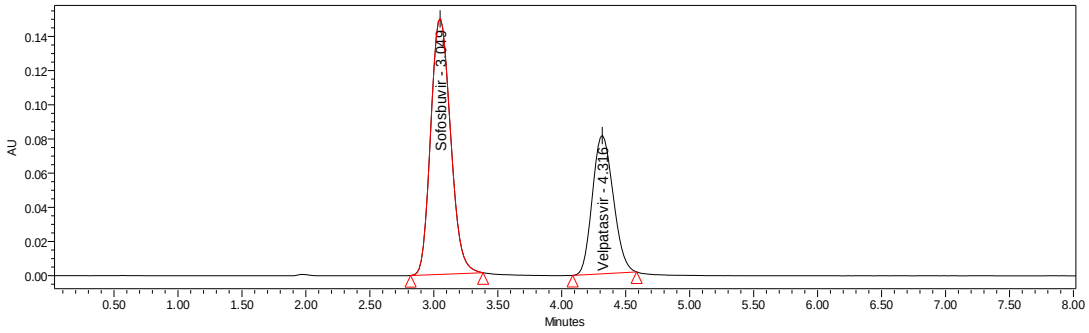


Fig 4.18 b Robustness Chromatogram (Mobile Phase Flow rate at 1.2 mL/min)

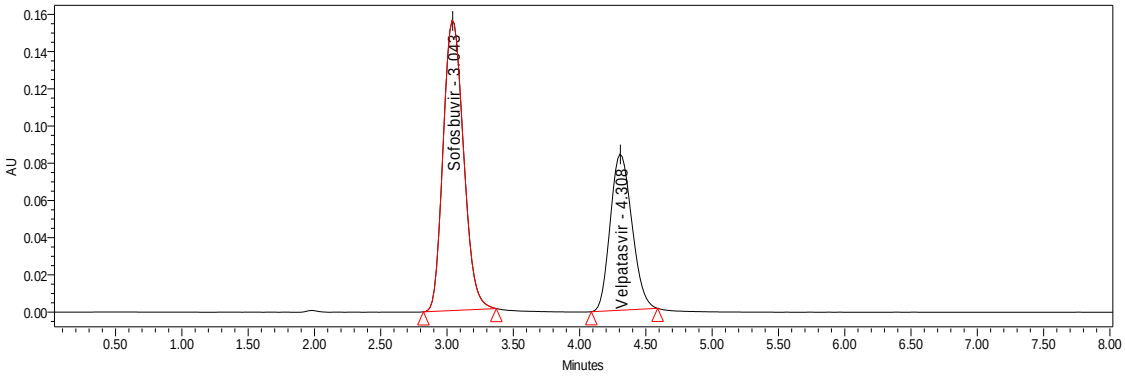


Fig 4.19 a Robustness Chromatogram at 20º C Column temperature.

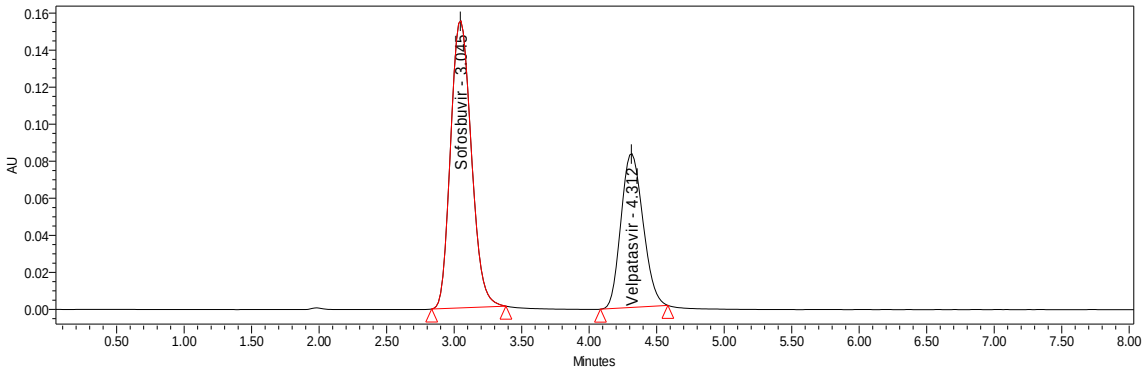


Fig 4.19 b Robustness Chromatogram at 30 ºC Column temperature.

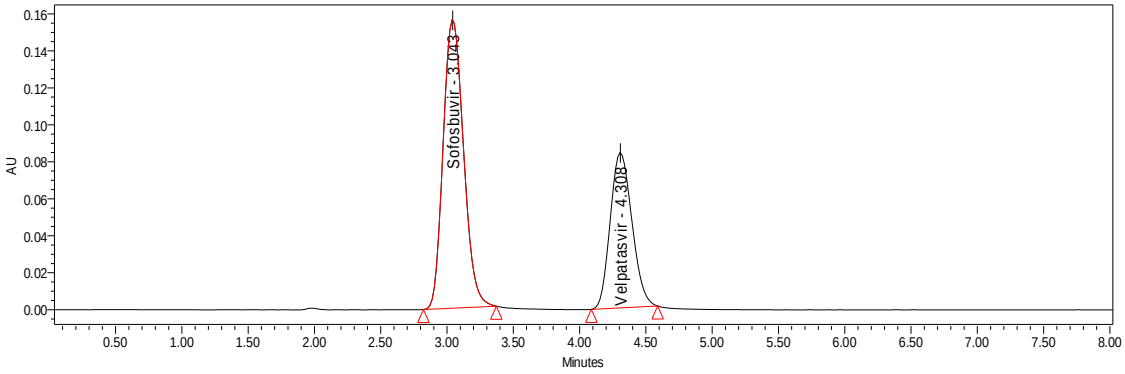


Fig 4.20 a Robustness Chromatogram at Mobile phase pH 3.1

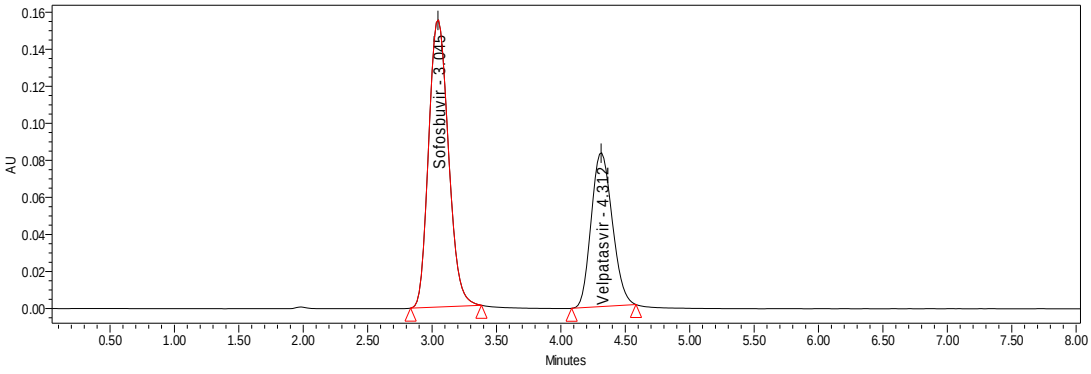


Fig 4.20 b Robustness Chromatogram at Mobile phase pH 3.3

Table- 4.11 Robustness Parameter of SOF and VEL

	Parameter	**Mobile phase flow rate. (mL/min)**			**Column temperature °C**			**Buffer pH**		
		0.8	**1.0**	**1.2**	**20**	**25**	**30**	**3.1**	**3.2**	**3.3**
SOF	Mean peak area (AU)	9416963	9436039	9456257	9376469	9454160	9546386	9341702	947503	9562431
	SD	1525.937	2597.38	4338.7	8493.9	4845.5	28271.8	17083	14889	13982.2
	% RSD	0.0162	0.0275	0.045	0.0905	0.0512	0.2961	0.1828	0.15714	0.1462
VEL	Mean peak area (AU)	321727.7	323472	324794.7	318496	32624.1	336844	314742	327722.3	332496.3
	SD	161.97	395.186	75.566	562.3	2826.4	2575.6	2486.2	2264.3	2845.466
	% RSD	0.0503	0.12217	0.0232	0.176	0.8663	0.7646	0.7899	0.6973	0.855789

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Result : Small and deliberate variations in the experimental conditions showed little or no change in the validation parameters of the method developed for the simultaneous estimation of SOF and VEL in SOFOSVEL tablet form. The % RSD for the Peak area were all within the acceptance range of less than 2.

All the validation parameters investigated for SOF and VEL combination tablet are incorporated in the Table 4.12.

TABLE 4.12 Table of Method Validation parameters of SOF and VEL.

S.No	Parameter	Value		Reference value
		SOF	**VEL**	
1	Theoretical plates (N)	11036.8	8372	More than 2000
2	Tailing factor (T)	1.18	1.28	Less than 2
3	Resolution (R)	----	4.34	More than 2
4	% RSD of Peak area	0.27	0.289	Less than 2
5	% RSD of RT	0.027	0.502	Less than 2
6	Range (µg/mL)	2 – 8	0.5 – 2.0	-
7	LOD (µg/mL)	0.475	0.65	-
8	LOQ(µg/mL)	1.44	1.98	-
9	Accuracy level (%)	(% Recovery)		98% - 102%
	50	99.78	99.92	
	100	99.97	99.94	
	150	100.02	99.97	
10	Method precision (% Assay)	99.56	99.87	98% -102%
11	Specificity	Specific	specific	Specific
	Robustness	%RSD (RT)		

12	(Experimental condition)	SOF			VEL			
	Mobile phase flow rate (mL/min)	0.016 (0.8)	0.027 (1.0)	0.045 (1.2)	0.05 (0.8)	0.122 (1.0)	0.02 (1.2)	Less than 2
	T (ºC)	0.0905 (20)	0.051 (25)	0.296 (30)	0.176 (20)	0.866 (25)	0.7646 (30)	
	Buffer pH	0.182 (3.1)	0.1571 (3.2)	0.1462 (3.3)	0.789 (3.1)	0.697 (3.2)	0.855 (3.3)	

All the above validation parameter values were found to be within acceptance range. While establishing the optimized condition Methanol was used in the mobile phase which is cheaper and easily available. The above method and investigation was much better in its RT values, Linearity, and Precision when compared to previously reported methods.