ORIGINAL ARTICLE

Development and Validation of Stability-indicating UPLC Method for the Simultaneous ESTIMATION of Voxilaprevir, Sofosbuvir, and Velpatasvir in Formulations

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Abstract

Aim: The aim of the present research work was to develop a sensitive, rapid and accurate, stability-indicating RP-UPLC method for the simultaneous estimation of voxilaprevir (VXR), sofosbuvir (SFR), and velpatasvir (VLR) in formulations. **Materials and Methods:** The chromatographic separation of mixture of VXR, SFR, and VLR was attained in isocratic method utilizing a mobile phase of 0.01N potassium dihydrogen orthophosphate (pH 4.8) and methanol in the proportion of 50:50% v/v utilizing a CHS C18 column which has dimensions of 100 × 2.1 mm, 2.0 m particle size and the flow rate of 1.0 mL/min. The detection system was monitored at 260 nm wavelength maximum with 1.0 mL injection volume. **Results:** The retaining time for VXR, SFR, and VLR was achieved at 1.468 min, 0.606 min, and 0.848 min, respectively. VXR, SFR, and VLR and their combined drug formulation were exposed to thermal, acidic, oxidative, photolytic, and alkaline conditions. The present method was validated as per the guidelines given by the ICH for specificity, accuracy, sensitivity, linearity, and precision. **Conclusion:** The developed method was highly sensitive, rapid, precise, and accurate than the earlier reported methods. The total run time was decreased to 3.0 min; hence, the technique was more precise and economical. Stability studies directed for the suitability of the technique for degradation studies of VXR, SFR, and VLR. The projected method can be utilized for routine analysis in quality control department in pharmaceutical trades.

Key words: RP-UPLC, sofosbuvir, stability, validation, velpatasvir, voxilaprevir

INTRODUCTION

Voxilaprevir (VXR), sofosbuvir (SFR), and velpatasvir (VLR) drugs were combined in a single dosage form (film coated tablet) in the brand name of Vosevi for the treatment of hepatitis-C. These three drugs will acts against hepatitis-C virus (HCV) in three different mechanisms.[1,2]

VXR produce its antiviral activity by binding reversibly and inhibiting the nonstructural protein (NS) 3/4A serine protease of HCV. Subsequently viral reproduction of HCV genetic material and translation into a single polypeptide, NS3, and its activating cofactor NS4A are responsible for splitting genetic components into the following nonstructural and structural proteins essential for gathering into mature virus: NS3, NS4A, NS4B, NS5A, and NS5B[2]. By inhibiting viral protease NS3/4A, VXR therefore prevents viral replication and function. VXR chemically designated as (1R, 18R, 20R, 24S, 27S, 28S)-N-[(1R, 2R)- 2-(Difluoromethyl) -1-{[(1-methylcyclo propyl) sulfonyl] carbamoyl} cyclopropyl]-28- ethyl-13,13-difluoro-7-methoxy -24-(2-methyl-2- propanyl)-22, 25-dioxo-2, 21-dioxa-4, 11, 23, 26 tetraaza pentacyclo dioxanonacosa -3(12), 4, 6, 8, 10-pentaene- 27-carboxamide with molecular weight of 868.94 g/ mole[3-5] [Figure 1].

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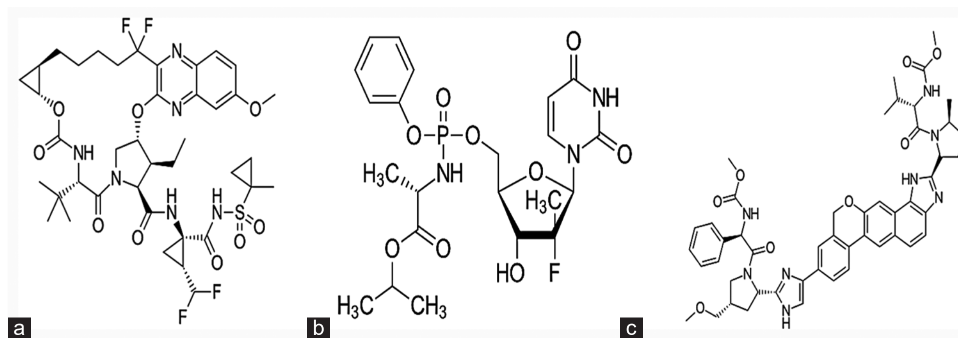

Figure 1: Structures of (a) voxilaprevir, (b) sofosbuvir, and (c) velpatasvir

SFR chemically designated as isopropyl(2S)-2-[(2R,3R,4R, 5R)-5-(2,4- dioxopyrimidin-1-yl)-4-Fluoro –hydroxy-4-methyl -tetrahydrofuran-2-yl]methoxy-phenoxy-phosphoryl] amino]propionate with molecular weight of 529.4 g/mole [Figure 1]. SFR prevents HCV NS-5B RNA-dependent RNA polymerase and acts as a chain terminator. Specifically it also inhibits HCV viral replication by binding to the two Mg^{+2} ions present in HCV NS5B polymerases.[2,6,7]

VLR is a selective NS-5A inhibitor which binds to domain-I of NS-5A comprising amino acids 33–202[1]. This NS-5A inhibiting component competes with RNA for binding at this site. Inhibition of NS-5A is also known to induce redistribution of the protein to lipid droplets. The exact role of NS-5A in RNA replication is not yet understood although it is known to be an important constituent. VLR is chemically designated as Methyl{(2S)-1-[(2S,5S)-2-(9-{2-[(2S,4S)-1-{(2R)-2-[(methoxycarbonyl)amino]-2-phenylacetyl]-(methoxymethyl)-2-pyrrolidinyl]-1H- imidazol-4-yl}-1,-11-dihydroisochromeno[4', 3:'6,7]naphtha[1,2-d] imidazol -2-yl)-5-methyl-1-pyrrolidinyl]-3-methyl-1-oxo-2-butanyl} carbonate with molecular weight of 883.02 g/ mole[8-10] [Figure 1].

The literature review discloses that a very few spectroscopic,[7,8] LC-MS/MS,[9] and high performance liquid chromatographic techniques[10-16] have been reported for the estimation of VXR, SFR, and VLR. Based on the reported HPLC methods, there is a need to develop a rapid, sensitive reversed-phase-UPLC method for simultaneous estimation of VXR, SFR, and VLR in bulk and formulations.

MATERIALS AND METHODS

Chemicals and reagents

The standard components of VXR, SFR, and VLR were provided as a gift sample from spectrum Pharma Research Solutions, Hyderabad. VOSEVI tablets labeled to contain VXR 100 mg, SFR 400 mg, and VLR 100 mg were procured from the local market. HPLC grade acetonitrile and methanol were obtained from A.B enterprises, Mumbai, India. Orthophosphoric acid was bought from Ranchem, Mumbai, India. HPLC grade water was processed by utilizing Milli-Q Millipore water purification system used during the method development.

Liquid chromatography

Chromatographic system of waters UPLC system furnished with photodiode array detector, auto-sampler, and CHS C18 column which has dimensions of 100 × 2.1 mm, 2.0 m particle size. The output signal was monitored and integrated utilizing water Empower-2.0 software. The isocratic mobile consisting of 0.01 N Potassium dihydrogen ortho phosphate (pH 4.8) and methanol in the proportion of 50:50% v/v, pumped through the CHS C18 (100 × 2.1 mm, 2.0 m) column at a fixed flow of 1 mL/min. The injection volume of 1.00 mL was utilized to measure the chromatograms at 260 nm as wavelength maximum in the detection system.

Preparation of buffer

Accurately weighed 1.36 g of potassium dihydrogen orthophosphate in a 1000 mL of volumetric flask add about 900 mL of milli-Q water added and degas to sonicate and finally make up the volume with water then added 1 mL of triethylamine then PH adjusted to 4.8 with dilute orthophosphoric acid solution.

Preparation of stock and standard solution

Accurately weighed and transferred 10 mg of SFR and 2.5 mg of VLR and 2.5 mg of VXR working standards into a 25 mL clean and dry volumetric flask, add 3/4th volume of diluent (water:acetonitrile [50:50 v/v]), sonicated for 5 min and made up to final volume with diluent. 1.0 mL from the above stock solution was taken into a 10 mL volumetric flask and made up to 10 mL to get 40 µg/mL of SFR, 10 µg/mL of VLR, and 10 µg/mL of VXR.

Preparation of sample solution

Five tablets were weighed and calculated the average weight of tablets and then the weight equivalent to one tablet was

transferred into a 100 mL volumetric flask containing 50 mL of diluent and sonicated for 25.0 min. Further, the volume made up with diluent and subjected for filtration. From the filtrate, 1.0 mL solution was pipetted out into a 10.0 mL volumetric flask and made up to 10.0 mL with diluent.

Analytical method validation

The developed method for VXR, SFR, and VLR was subjected for validation for the parameters like limit of detection (LOD), limit of quantification (LOQ), linearity, robustness, precision, system suitability, and accuracy as per the guidelines the of ICH.[17-22]

RESULTS

Optimized chromatographic conditions

After systematic trials with different mobile phase compositions and other parameters involved in the technique, the following chromatographic conditions were employed:

- Mobile phase: 0.01N KH_2PO_4: Methanol (50:50 v/v)
- Flow rate: 1.0 mL/min
- Column: CHS C18 100 × 2.1 mm 2.0 m.
- Detector wave length: 260 nm
- Column temperature:30°C
- Injection volume: 1.00 mL
- Run time: 3.0 min
- Diluent: Water:Acetonitrile (50:50 v/v).

Specificity

It is the ability of a method to unequivocally evaluate the analyte components in the presence of other components such as impurities, degradants, and excipients expected to be present. This parameter was estimated by injecting and evaluating the blank, placebo, standard and sample solutions, and chromatograms, respectively.[18,21] Chromatograms of blank, placebo, and sample solutions are shown no peaks at the retaining time of VXR, SFR, and VLR peaks. The chromatograms of VXR, SFR, and VLR of standard, blank, formulation, and placebo are represented in Figure 2.

Linearity

Aliquots of 0.25, 0.50, 0.75, 1.0, 1.25, and 1.50 mL of standard stock solution were pipetted out from the standard stock solution of concentration 100 µg/mL of VXR, 400 µg/mL of SFR, and 100 µg/mL of VLR and made up to 10.0 mL mark with diluent. The resulting solutions were came into 2.5–15.0 µg/mL of VXR, 10.0–60 µg/mL of SFR, and 2.5–15.0 µg/mL of VLR concentration range. The resulting linearity solutions were infused into a chromatographic system and form the chromatograms linearity graph was plotted by taking the peak area on Y-axis and concentration on X-axis.[22,23] The calibration graphs are shown in Figures 3-5 and Table 1.

System suitability

Six replicates of the standard reference solution were processed and infused to perform the system suitability parameter and the resulting chromatograms peak area, retention time, resolution, plate count, and tailing were measured.[24] The findings of system suitability parameter are shown in Table 2 and related chromatograms are given in Figure 2c.

LOD and LOQ

LOD and LOQ parameters for VXR, SFR, and VLR were calculated form the linear regression equation. Linearity

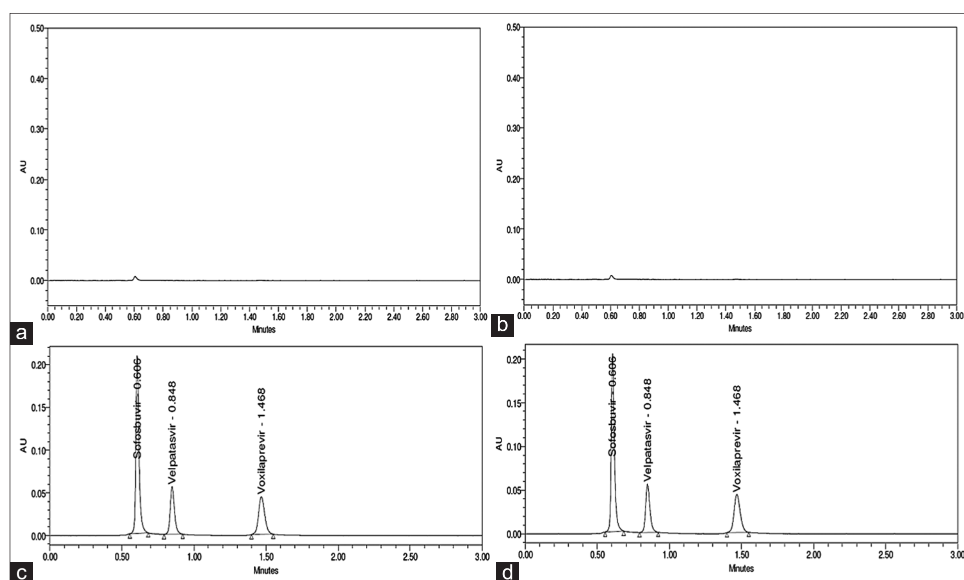

Figure 2: Chromatograms of (a) blank, (b) placebo, (c) standard, and (d) formulation

Table 1: Calibration curve data of VXR, SFR, and VLR

SFR		VLR		VXR	
Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area
10	80,570	2.5	29,134	2.5	31,001
20	154,713	5	57,385	5	60,744
30	235,700	7.5	84,709	7.5	91,775
40	311,047	10	113,309	10	120,409
50	388,511	12.5	143,371	12.5	150,447
60	471,096	15	170,419	15	183,558
Regression equation					
y=7805.4x+358.26		y=11367x+226.54		y=12132x+144.43	
Correlation coefficient (R^2)					
0.9999		0.9999		0.9998	

VXR: Voxilaprevir, SFR: Sofosbuvir, VLR: Velpatasvir

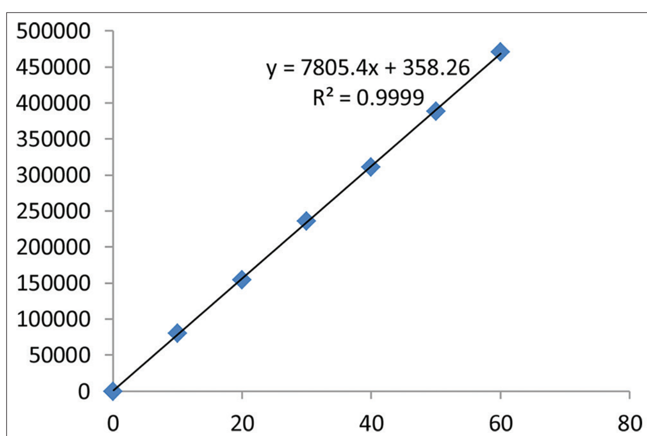

Figure 3: Linearity of sofosbuvir

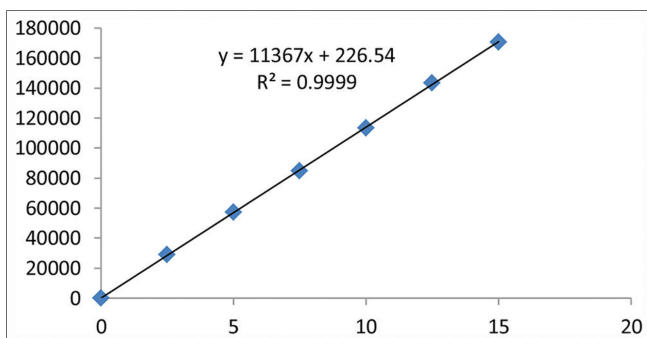

Figure 4: Linearity of velpatasvir

values, graph, and regression equation were got from the linearity study and the LOD and LOQ values are represented in Table 3.

Precision

Analytical method precision is defined as closeness of agreement between the replicate measurements of the analyte. It is expressed as the percentage coefficient of correlation or relative standard deviation (RSD) of the replicate measurements.

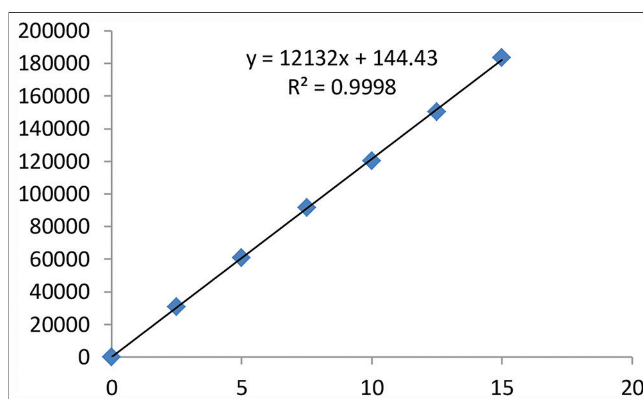

Figure 5: Linearity of voxilaprevir

System precision

Working standard preparation of 1.0 µL solution was infused 6 times into the chromatographic system and chromatograms were obtained. %RSD of the peak area was calculated. The findings of system precision are shown in Table 4.

Method precision

Working sample solutions of 1.0 µL were infused 6 times into the chromatographic system and chromatograms were obtained. The %RSD of the assay result of six preparations was determined. The findings achieved for assay are represented in Table 5.

Intermediate precision

Working standard preparation of 1.0 µL was infused 6 times test preparations into the chromatographic system and

Table 2: VXR, SFR, and VLR system suitability results

S. No.	Peak name	Peak area	Retention time	Plate count	Resolution	Tailing
1.	SFR	312779	0.606	4198		1.03
2.	VLR	112479	0.848	4247	5.7	1.25
3.	VXR	120173	1.468	5363	8.5	1.11

VXR: Voxilaprevir, SFR: Sofosbuvir, VLR: Velpatasvir

Table 3: Limit of detection and limit of quantification results

Parameter	Measured concentration (µg/mL)		
	Sofosbuvir	Velpatasvir	Voxilaprevir
Limit of detection	0.13	0.01	0.01
Limit of quantification	0.40	0.02	0.02

Table 4: System precision data

S. No.	Peak area response of drugs		
	SFR	VLR	VXR
1.	312,069	112,479	120,173
2.	312,779	113,408	121,013
3.	312,238	110,728	120,615
4.	312,774	113,342	120,586
5.	311,121	113,450	120,450
6.	307,430	113,258	120,670
Average	311,402	112,778	120,585
STDV	2038.5	1066.7	275.4
% RSD	0.7	0.9	0.2

STDV: Standard deviation, RSD: Relative standard deviation

chromatograms were obtained. The %RSD was evaluated for peak areas. The findings of intermediate precision study are represented in Table 6.

Accuracy

A known amount of VXR, SFR, and VLR at each three concentration levels of 50%, 100%, and 150% was added to a pre-analyzed sample solution and injected in triplicate at each level into the chromatographic system. The mean percentage recovery of VXR, SFR, and VLR at each level was estimated. The findings are represented in Table 7.

Robustness

Working standard solution prepared as per test method was infused into the chromatographic system at variable conditions such as flow rate at ±0.1 mL/min, mobile organic phase composition by ±10%, and column temperature by ±5°C. The results of robustness study parameter such as peak area, retention time, plate count, and tailing factor were within the limits.

Table 5: Method precision results

S. No.	Peak area response of drugs		
	SFR	VLR	VXR
1.	314,292	113,184	120,333
2.	311,541	112,188	121,233
3..	311,924	113,675	120,563
4.	312,765	113,015	120,619
5.	310,377	112,819	120,173
6.	313,315	112,899	120,932
Average	312,369	112,963	120,642
STDV	1385.7	486.6	388.7
% RSD	0.4	0.4	0.3

STDV: Standard deviation, RSD: Relative standard deviation

Table 6: Intermediate precision results

S. No.	Peak area response of drugs		
	SFR	VLR	VXR
1.	310,514	108,619	117,972
2.	310,229	109,543	115,023
3.	306,881	108,648	116,751
4.	307,498	107,947	117,905
5.	301,224	109,878	118,239
6.	302,657	108,905	116,389
Average	306,501	108,923	117,047
STDV	3840.9	695.3	1235.3
% RSD	1.3	0.6	1.1

Forced degradation studies

Acid degradation studies

To 1 mL of stock solution VXR, SFR, and VLR, 1 mL of 2N hydrochloric acid was added and refluxed for 30 min at 60°C.[22-24] The resultant solution was diluted to obtain 40 µg/mL of SFR and 10 µg/mL of VLR and 10 µg/mL of VXR solution and 1.0 µL was injected into the chromatographic system and the chromatograms were recorded to assess the stability of sample [Figure 6 and Table 8].

Table 7: Percentage recovery results

Spiked level (%)	SFR				VLR				VXR			
	Spiked (µg/mL)	Recovery (µg/mL)	% Recovery	Mean % recovery	Spiked (µg/mL)	Recovery (µg/mL)	% Recovery	Mean % recovery	Spiked (µg/mL)	Recovery (µg/mL)	% Recovery	Mean % recovery
50	20	19.9527	99.76	99.90	5	4.964415	99.29	99.87	5	4.969964	99.40	99.81
	20	20.15859	100.79		5	4.988168	99.76		5	4.999637	99.99	
	20	20.11874	100.59		5	4.988255	99.77		5	4.980762	99.62	
100	40	40.14655	100.37		10	9.950955	99.51		10	9.977629	99.78	
	40	39.68761	99.22		10	9.99415	99.94		10	9.944576	99.45	
	40	40.02432	100.06		10	9.975939	99.76		10	10.03351	100.34	
150	60	59.5714	99.29		15	15.05265	100.35		15	14.92924	99.53	
	60	59.66109	99.44		15	15.07553	100.50		15	15.102	100.68	
	60	59.73732	99.56		15	14.98993	99.93		15	14.929	99.53	

Oxidation

To 1 mL of stock solution of VXR, SFR, and VLR, 1 mL of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60°C. For UPLC study, the resultant solution was diluted to obtain 40 µg/mL of SFR and 10 µg/mL of VLR and 10 µg/mL of VXR solution and 1.0 µL was injected into the chromatographic system and the chromatograms were recorded to assess the stability of sample [Figure 7 and Table 8].

Alkali degradation studies

To 1 mL of stock solution VXR, SFR, and VLR, 1 mL of 2N sodium hydroxide was added and refluxed for 30 min at 60°C. The resultant solution was diluted to obtain 40 µg/mL of SFR and 10 µg/mL of VLR and 10 µg/mL of VXR solution and 1.0 µL was injected into the chromatographic system and the chromatograms were recorded to assess the stability of sample [Figure 8 and Table 8].

Dry heat degradation studies

The standard drug solution was placed in oven at 105°C for 6 h to study dry heat degradation.[24] For UPLC study, the resultant solution was diluted to get 40 µg/mL of SFR and 10 µg/mL of VLR and 10 µg/mL of VXR solution and 1.0 µL was injected into the chromatographic system and the chromatograms were recorded to assess the stability of sample [Figure 9 and Table 8].

Photo stability studies

The photochemical stability of the drug was also studied by exposing the (100 µg/mL, 400 µg/mL, and 100 µg/mL) solution to UV Light by keeping the beaker in UV Chamber for 3 days or 200 Watt hours/m^2 in photostability chamber. For UPLC study, the resultant solution was diluted to obtain 40 µg/mL of SFR and 10 µg/mL of VLR and 10 µg/mL of VXR solution and 1.0 µL was injected into the chromatographic system and the chromatograms were recorded to assess the stability of sample [Figure 10 and Table 8].

Neutral degradation studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6 h at a temperature of 60°C. For UPLC study, the resultant solution was diluted to obtain 40 µg/mL of SFR and 10 µg/mL of VLR and 10 µg/mL of VXR solution and 1.0 µL was injected into the chromatographic system and the chromatograms were recorded to assess the stability of sample [Figure 11 and Table 8].

Table 8: Results of stress degradation study

S. No.	Degradation condition	SFR		VLR		VXR	
		% Recovery	% Degraded	% Recovery	% Degraded	% Recovery	% Degraded
1.	Acid hydrolysis	94.10	5.90	94.17	5.83	95.28	4.72
2.	Base hydrolysis	95.56	4.44	95.69	4.31	96.36	3.64
3.	Peroxide	96.81	3.19	96.90	3.10	96.42	3.58
4.	Dry heat	97.12	2.88	97.74	2.26	97.04	2.96
5.	Photo stability	98.82	1.18	98.42	1.58	98.49	1.51
6.	Water sample	99.42	0.58	99.10	0.90	99.68	0.32

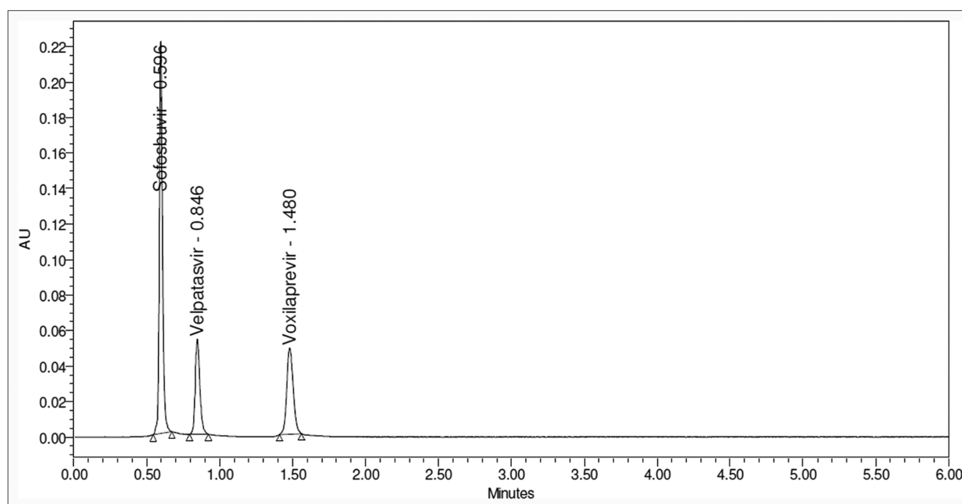

Figure 6: Chromatogram for acid degradation study

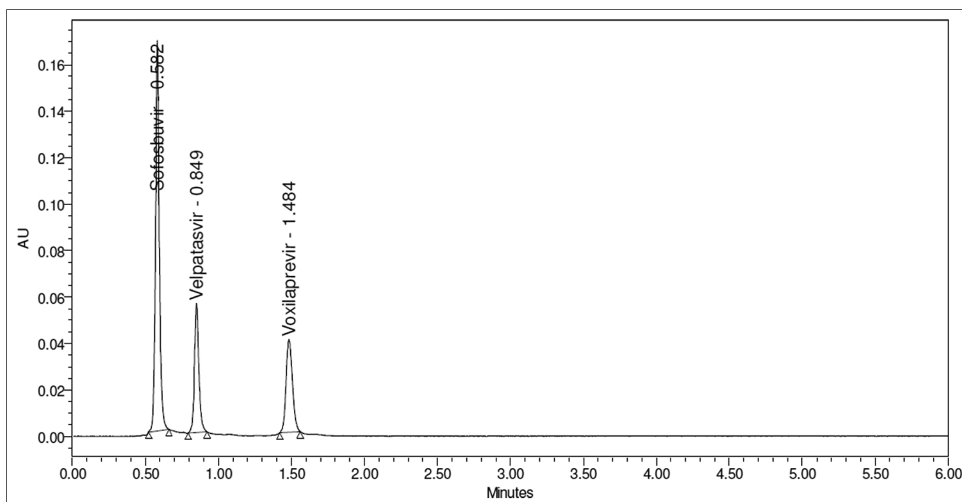

Figure 7: Chromatogram for oxidation degradation study

Assay of marketed formulation

The marketed formulation of VOSEVI (film coated tablet) was evaluated by infusing 1.0 µL of reference and analyte solutions 6 times into the chromatographic system and the resulting chromatograms of analytes were documented. The quantity of analytes existed in the marketed formulation was estimated by equating the peak area of reference and analyte. The % assay of VXR, SFR, and VLR was found to be 99.0–101.0%.

DISCUSSION

In the literature, all the methods were reported on the HPLC techniques with more retention time and run times.

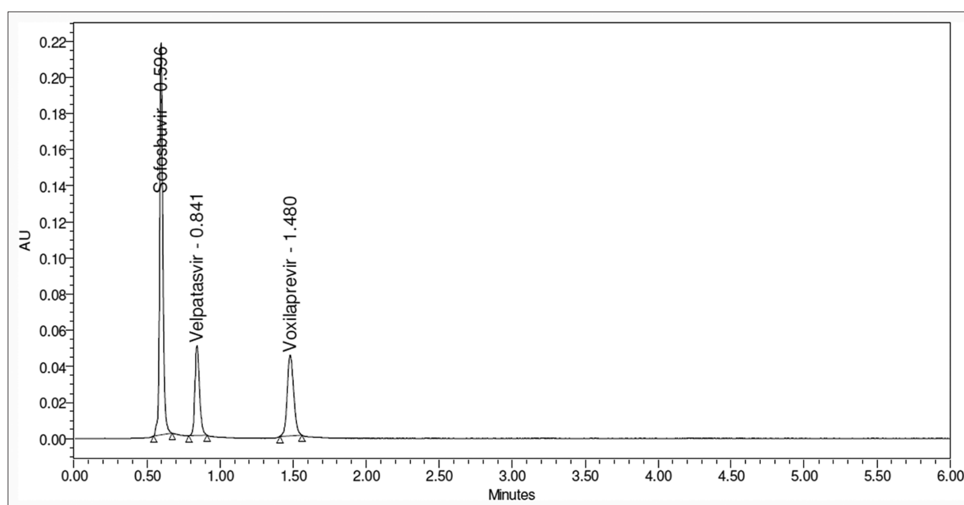

Figure 8: Chromatogram for alkali degradation study

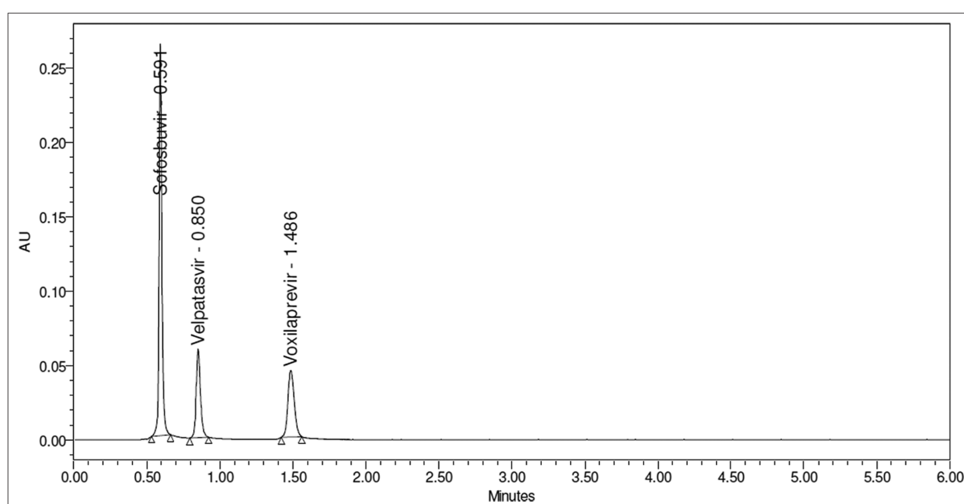

Figure 9: Chromatogram for dry heat degradation study

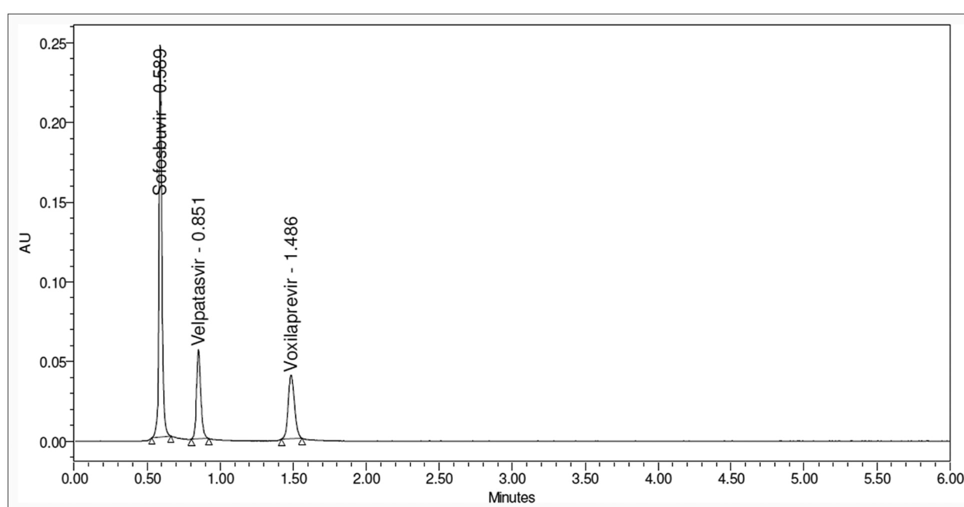

Figure 10: Chromatogram for photostability study

In the present work, we selected UPLC to reduce the total run time. Method development was executed with different columns and mobile phases. Finally, the method was optimized with mobile phase of 0.01N potassium

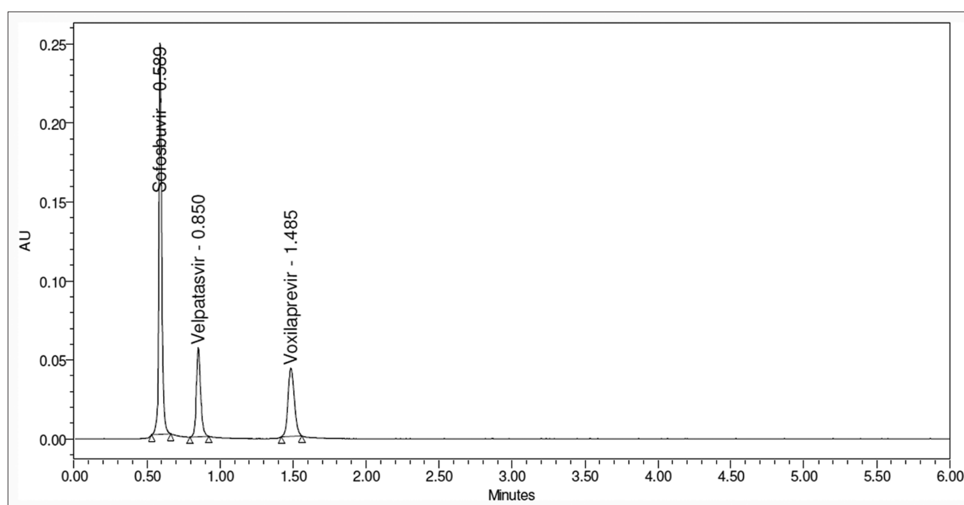

Figure 11: Chromatogram for neutral degradation study

dihydrogen orthophosphate (pH 4.8) and methanol in the proportion of 50:50% v/v utilizing a CHS C18 column which has dimensions of 100 × 2.1 mm, 2.0 m particle size, and the flow rate of 1.0 mL/min. Further, the developed method was subjected for validation and forced degradation studies. Validation was executed as per the ICH Q2R1 guidelines for the parameters specificity, linearity, system suitability, LOD and LOQ, precision, accuracy, and robustness. All the parameters were within the limits. Developed method was subjected for forced degradation studies as per the ICH such as neutral degradation, photostability, dry heat degradation, alkali degradation, oxidation, and acid degradation. The degradation results also produced in the results section.

CONCLUSION

A sensitive, rapid, and accurate, stability-indicating RP-UPLC method for the simultaneous estimation of VXR, SFR, and VLR in formulations was developed and validated as per the ICH guidelines. Retention times for VXR, SFR, and VLR were achieved at 1.468 min, 0.606 min, and 0.848 min, respectively. Mean percentage recovery of VXR, SFR, and VLR was found to be 99.90%, 99.87%, and 99.91%, respectively. LOD and LOQ values obtained from regression equations of VXR, SFR, and VLR and were found to be 0.01 µg/mL/0.02 µg/mL, 0.13 µg/mL/0.40 µg/mL, and 0.01 µg/mL/0.02 µg/mL. Regression equation of VXR, SFR, and VLR was: y = 12132x+144.43, y = 7805.4x+358.26, and y = 11367x+226.54, respectively. Stability studies of these drugs proven that the percentage degradation of analytes was found in between 0.32% and 5.90%. Retention time and total run times of analytes were decreased. Hence, the developed method was rapid and economical that can be applicable in routine analysis of these drugs in quality control department of pharmaceutical trades.

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