Journal of Pharmaceutical Research International

26(2): 1-10, 2019; Article no.JPRI.40460
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

A Novel Stress Indicating RP-HPLC Method Development and Validation for the Simultaneous Estimation of Velpatasvir and Sofosbuvir in Bulk and Its Tablet Dosage Form

D. Chinababu[1*]

[1]Santhiram College of Pharmacy, Nandyal, Andhra Pradesh, India.

Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/JPRI/2019/v26i230135
Editor(s):
(1) Dr. Stefano Manfredini, Professor of Pharmaceutical Chemistry and Toxicology, Department of Life Sciences and Biotechnology, Faculty of Medicine Pharmacy and Prevention, University of Ferrara, Ferrara, Italy.
(2) Dr. Mohamed Fathy Mohamed Ibrahim, Professor, Department of Pharmaceutics, Faculty of Pharmacy, Assiut University, Assiut, Egypt.
Reviewers:
(1) Dr. Minaketan Sahoo, Department of Pharmaceutical Analysis & Quality Assurance, Institute of Pharmacy & Technology, India
(2) Ana Carolina Kogawa, Department of Pharmaceutics, School of Pharmaceutical Sciences, Universidade Estadual Paulista, Araraquara, Brazil.
Complete Peer review History: http://www.sdiarticle3.com/review-history/40460

Original Research Article

Received 03 January 2018
Accepted 18 April 2018
Published 28 March 2019

ABSTRACT

Aim: The objective of the study was simplest, accurate, precise and robust reversed phase high performance liquid chromatographic (RP-HPLC) method was developed for the estimation of Velpatasvir (VEL) and Sofosbuvir (SOF) in the bulk and its tablet dosage form.
Study Design: The Quantitative and Qualitative estimation and designed forced degradation study of Velpatasvir & Sofosbuvir by RP-HPLC.
Place and Duration of Study: The study was carried at Santhiram College of Pharmacy and time taken 4 months.
Method: The method was attained by used Waters(5µm, C18 250 x 4.6 mm) column with mobile phase consists of 0.5 mM disodium hydrogen phosphate buffer pH adjusted to 6.5, with Orthophosphoric acid and Methanol in the ratio of 78:22 v/v, a flow rate of 1.0 mL/min and ultraviolet detection at 285 nm.
Results: The method was validated as per ICH guidelines with different parameters, the mean retention times of VEL and SOF were found to be 2.8 & 4.7 min respectively. The resolution between VEL and SOF was found to be 10.66. The Correlation coefficients for calibration curves

**Corresponding author: E-mail: chinababu.rao@gmail.com;*

within the detection range of 32.5 - 97.5 and 125 - 375 µg/mL were 0.999 for VEL and SOF respectively. The LOD and LOQ for VEL and SOF were found to be 0.0068-0.029 µg/mL and 0.104-0.342 µg/mL respectively.

Conclusion: The results were indicated that the developed method was used for the routine analysis of VEL & SOF combined form in bulk and its commercial formulation. To the best of our knowledge, there was no method of RP-HPLC for the determination of VEL alone or in combination with SOF molecule.

Keywords: Velpatasvir; sofosbuvir; HPLC; commercial formulations.

1. INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health challenge nowadays. It has been estimated that the global prevalence of HCV infection is around 2%, with 170 million persons chronically infected with the virus and 3 to 4 million persons newly infected each year [1,2].

It is a pathogen that is already responsible for a significant proportion of liver disease in various regions of India [3].

Velpatasvir (VEL) is a novel HCV nonstructural Protein 5A (NS5A) inhibitor that was developed in combination with other drugs, which are directly acting antiviral for the treatment of HCV infections [4]. The IUPAC name for velpatasvir is Methyl {(1R)-2-((2S,4S)-2-(5-{2- ((2S,5S) -1-{(2S)-2 -((methoxycarbonyl) amino)-3- methyl butanoyl} -5-methyl pyrrolidin-2-yl)- 1,11 dihydro(2) benzopyrano (4',3':6,7) naphtha (1,2-d) imidazol-9-yl}-1H –imidazol -2-yl) -4- (methoxy methyl) pyrrolidin-1-yl)-2-oxo-1-phenylethyl} carbamate. It is a white to off-white powder, slightly soluble in water. It has a molecular formula of $C_{49}H_{54}N_8O_8$ [5].

Sofosbuvir SOF is a nucleotide pro-drug that effectively inhibits 1-6 HCV RNA replicons in vitro and has proved to have a high sustained virologic response (SVR) rates [5,6]. Sofosbuvir is a prodrug of 2'-deoxy-2'-fluoro-2'-C-methyluridine monophosphate that is phosphorylated intra cellularly to the active triphosphate form [7]. Chemically it is (S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5- (2,4- dioxo-3,4-di hydro pyrimidine-1 (2H)-yl) -4- fluoro-3-hydroxy-4-methyl tetrahydrofuran-2-yr)methoxy)(phenoxy)phosphorylamino)propano ate. It is a white to off-white crystalline powder, found to be slightly soluble in water and freely soluble in alcohol and acetone. It has a molecular formula of $C_{22}H_{29}FN_3O_9P$ [8]. Chemical structures of VEL and SOF were shown in Figs.1 and 2 respectively.

The combined dosage form (Tablet – Velpanat, Natco Pharma) consists of 100 mg of VEL and 400 mg of SOF was indicated for the treatment of chronic hepatitis C virus (HCV) infection in adults [4,6]. LC-MS/MS method has been reported for the estimation of SOF with Ledipasvir in human plasma [9]. Two UPLC-MS/MS methods have been reported for determination of SOF [10] and in a combination of Ledipasvir [11,12] in human plasma for determination of bioequivalence studies. Few RP-HPLC methods have been reported for the estimation of SOF alone [12,13] or with a combination of other drugs like Ledipasvir [14,15] and Simeprevir used in the combination for the treatment of HCV infection [16].

2. EXPERIMENTAL

2.1 Reagents

All the chemicals and reagents were of analytical grade. Water was redistilled and filtered with a membrane filter. Methanol – HPLC grade (Merck, India), Ortho phosphoric acid and disodium hydrogen phosphate (SD fine chem, India) were used to prepare mobile phase. Pharmaceutical grade standard drugs viz., Velpatasvir and Sofosbuvir were kindly gifted by Natco Pharma Ltd, Hyderabad, India. The combined tablet formulation contains 100 mg of Velpatasvir and 400 mg of Sofosbuvir (Velpanat, Natco) purchased from local market of Nellore.

2.2 Chromatographic Conditions

The method was developed by using HPLC system consisted of an LC Waters (Waters, Milford, MA, USA) using a Water's C_{18} 250 x 4.6 mm, 5µm column, a quaternary gradient system (600 Controller), in line degasser (Waters, model AF). The system was equipped with a photodiode array detector (Waters, 2998 model) and auto sampler (Waters, model 717 plus).

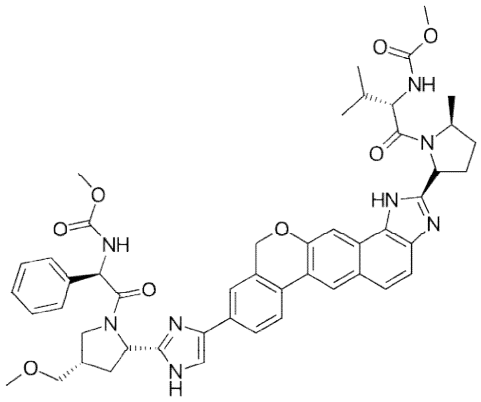

Fig. 1. Chemical structure of velpatasvir

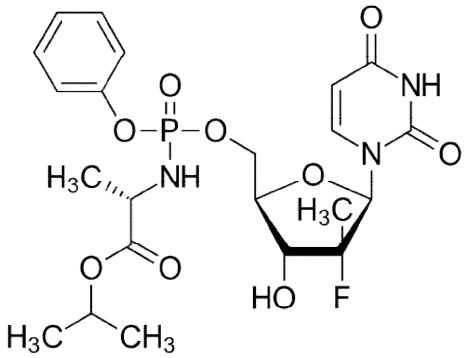

Fig. 2. Chemical structure of Sofosbuvir

Data were processed using Empower Pro software (Waters, Milford, MA, USA). The Isocratic mobile phase consists of a mixture of 0.5 mM disodium hydrogen phosphate buffer pH adjusted to 6.5, with Ortho phosphoric acid and Methanol in the ratio of 78:22% v/v was used throughout the analysis. The mobile phase was pumped at a flow rate of 1.0 mL/min. UV detection wavelength for analytes was 285 nm. The column temperature was kept ambient and the injection volume was 10µL.

2.3 Solution Preparation

2.3.1 Standard stock solution preparation

10 mg of VEL and SOF each was weighed accurately and transferred to individual 10 ml volumetric flasks. Dissolved and diluted with methanol to get a concentration of 1000 µg/ml.

2.3.2 Working standard solution

1.625 mL of VEL and 6.25 mL of SOF standard stock solutions were accurately measured and transferred to a 25 mL volumetric flask, mixed well and diluted to final volume with a diluent, so as get the final concentrations of 65 µg/mL of VEL and 250 µg/mL of SOF.

2.3.3 Sample solution preparation (Assay)

Twenty tablets were weighed and finely powdered. The average weight of tablets was determined. A portion of powder was weighed equivalent to 10 mg of VEL and transferred to a 10 mL volumetric flask. 10 mL of methanol was added to disintegrate tablets completely by using ultra sonicator for 10 min and solution concentration was 1000 µg/mL.

2.3.4 Working sample solution

The solution was further diluted to get final concentrations of 65 µg/mL of VEL and 250 µg/mL of SOF. This solution was filtered through 0.45 µm membrane filter. The 10µL of this solution was injected into HPLC system.

2.4 Method Validation

The method was validated according to ICH guidelines for the estimation of velpatasvir and sofosbuvir. The following validation parameters are enveloped precision, accuracy, linearity, limit of detection & limit of quantification, robustness and forced degradation studies. The standard solution was prepared at six concentrations ranging from 32.5- 97.5µg/mL for VEL and 125-375 µg/mL for SOF solutions were prepared for linearity. The regression of the curve was obtained by peak area vs concentration. The method sensitivity was measured by the limit of detection and limit of quantification. The limit of detection and limit of quantification were determined by signal to noise ratio 3:1& 10:1.The precision of the method was assessed by measured six times standard solution of VEL & SOF and measured the area of all six injections in the HPLC chromatographic system. The accuracy of the method was determined by standard addition and recovery method. The accuracy of the method was evaluated in triplicate at three concentration levels, i.e. 50%, 100% and 150% of target test concentration and the percentages of recoveries were calculated. The robustness of the method was manifested by deliberate changes in experimental conditions. The changes made in the chromatographic conditions like flow rate by ±0.2 mL/min, mobile phase composition of organic phase change ± 3 and the column temperature ±5 °C. The drugs were subjected to different stress conditions like acid (refluxed 0.1N HCl for 1 hr at 80°C), base (refluxed 0.1N NaOH for 4 hrs at 80°C), H_2O_2(stored 3% H_2O_2 room temp for 2 hrs) light and water near UV ≥200 FOR 10 days) forced degradation studies were conducted on the VEL & SOF.

3. RESULTS AND DISCUSSION

3.1 Method Development and Optimization of Chromatographic Conditions

During the optimization of the method, different columns (Inertsil C8, 250 mm×4.6 mm, 5 µm; Zorbax C18 250 mm×4.6 mm, 5 µm; Symmetry C18 250 mm×4.6 mm, 5 µm) and two organic solvents (acetonitrile and methanol) were tested. The chromatographic conditions were also optimized by using different buffers like phosphate, acetate and citrate for mobile phase preparation. After a series of screening experiments, it was concluded that phosphate buffers gave better peak shapes than their acetate and citrate counterparts. With acetonitrile as solvent both the peaks shows less theoretical plates and more retention time compared to methanol. The chromatographic separation was achieved on a Waters C18, 250 mm×4.6 mm, 5µm column, by using a mixture of 0.5 mM disodium hydrogen phosphate buffer pH adjusted to 6.5, with Ortho phosphoric acid and Methanol in the ratio of 78:22 v/v, as the mobile phase. The temperature was maintained ambient to facilitate mass exchange with the corresponding decrease of peak broadening and increase in sensibility. The flow rate kept was 1.0 mL/min to achieve adequate retention time of two peaks 2.80 min and 4.78 min for VLE and SOF respectively. Figs. 3, 4 and 5 shows blank, standard and sample chromatograms. The Table1 shows the optimized chromatographic conditions.

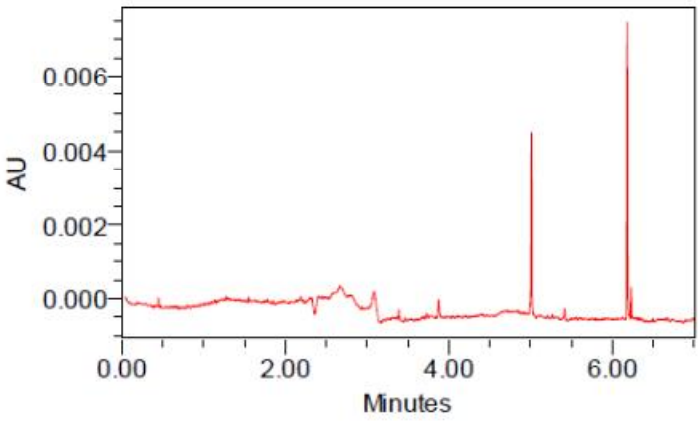

Fig. 3. Blank chromatogram

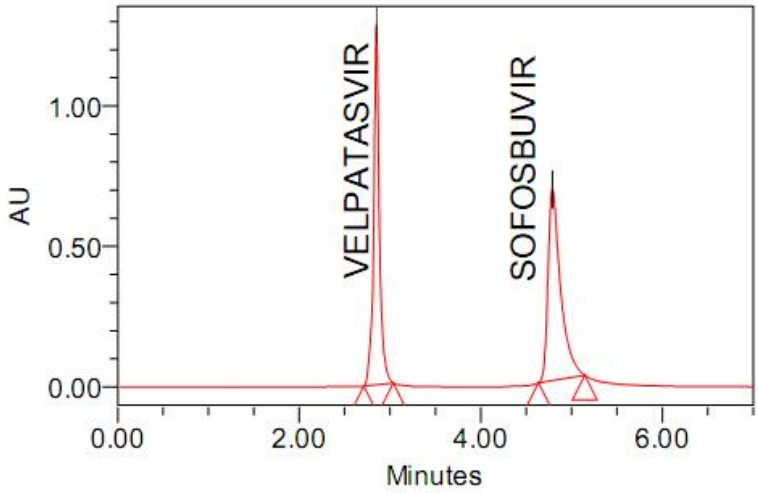

Fig. 4. Standard chromatogram of VEL & SOF

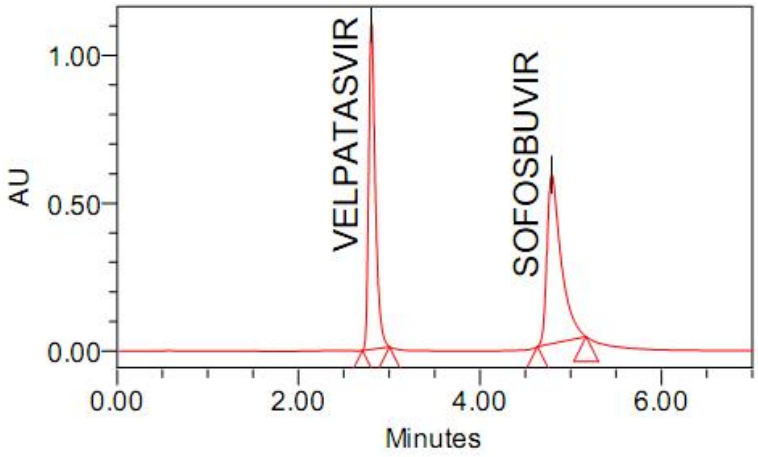

Fig. 5. Sample chromatogram of VEL & SOF

Table 1. Optimized HPLC conditions for simultaneous estimation of velpatasvir and sofosbuvir

S. No	Parameter	Description/Value
1.	Stationary Phase	Water's C18 (250X4.6X5)
2	Mobile Phase	0.5 mM Disodium Phosphate buffer (pH 6.5, adjusted with OPA) and MeOH in the ratio of 78:22 v/v
3	Flow rate	1 mL/min
4	Detection Wavelength (Isosbestic Point)	285nm
5	Detector	Photo diode array
6	Injection	Autosampler -Waters, model 717 plus
7	Injection volume	10 µl
8	Column Temperature	Ambient
9	Run time	6 mins
10	Diluent	Methanol
11	Rt's	Velpatasvir: 2.806 min Sofosbuvir: 4.780 min

3.2 Method Validation

When a method has been optimized it must be validated before practical use. By following ICH guidelines for analytical method validation, Q2 (R1), the validation characteristics were addressed [17].

3.3 System Suitability

The system suitability test ensures the validity of the analytical procedure as well as confirms the resolution between different peaks of interest. All critical parameters tested met the acceptance criteria on all days. As shown in the chromatograms (Figs. 4 & 5), two analytes were eluted by forming symmetrical single peaks well separated from each other and from excipients. Table 2 shows the System Suitability results.

3.4 Linearity

For the construction of calibration curves, five calibration standard solutions were prepared over the concentration range of 32.5 – 97.5 µg/ml for VEL and 125.0 – 375 µg/mL for SOF. The results, summarized in Table 3, showed a good correlation between analytes peak area and concentration with r > 0.999 (n = 5). Linearity curve was shown in Figs. 6 and 7.

3.5 Precision

The assay was investigated with respect to repeatability and intra-day precision. The repeatability of the system (while keeping the operating conditions identical) was examined by injecting analyte solution with 6 replicate injections. The RSD values varied from 0.47 to 0.86% Showed, that the inter-day precision of the method was satisfactory. Table 4 shows the precision results.

3.6 Accuracy

To govern the accuracy of the proposed method, recovery studies have been performed, known amount of pure drug sample solution at three different concentration levels, ie, 50%, 100%,150% was calculated. Accuracy was calculated as a percentage of recovery. The accuracy results tabulated as 5.

3.7 Limit of Detection (Lod) and Limit of Quantification (Loq)

The Limit of detection and limit of quantification were considered as the signal- to- noise ratio 3:1 and 10:1 respectively. The limit of detection and limit of quantitation to be determined 0.0068 µg/ml & 0.029 µg/ml for VEL and 0.104 µg/ml & 0.347 µg/ml for SOF respectively.

Table 2. System suitability results

S. No	Parameters	Results		Limits
		Velpatasvir	Sofosbuvir	
1	RSD of peak area	0.20	0.86	<2 n ≥ 6
2	Retention times	2.849	4.786	-
3	RSD of retention time	0.56	0.89	<2 n ≥ 5
4	USP plate count	13196	6255	>2000
5	USP tailing factor	1.06	1.75	T<2
6	USP resolution	-	10.66	R >2

Table 3. Linearity results of VEL & SOF

S. No	Linearity level	Velpatasvir		Sofosbuvir	
		Concentration (µg/mL)	Peak Area	Concentration (µg/mL)	Peak Area
1	50	32.5	2813066	125	3077228
2	75	48.75	4253268	187.5	4629475
3	100	65	5613521	250	6143585
4	125	81.25	7052657	312.5	7688257
5	150	97.5	8406053	375	9285177
Slope		86064		24759	
Intercept		33568		25128	
R^2		0.9999		0.9999	

Table 4. Results of method precision

S. No	Velpatasvir		Sofosbuvir	
	Peak Area	% Assay	Peak Area	% Assay
1	5516391	100.55	6556728	100.84
2	5518106	100.58	6531198	100.44
3	5518136	100.58	6475888	99.59
4	5555471	101.26	6429678	98.88
5	5565122	101.43	6412642	98.62
6	5570645	101.53	6481227	99.67
Average	5540645.17	100.99	6481226.83	99.67
SD	25775.38	0.47	55834.05	0.86
%RSD	0.47	0.47	0.86	0.86

Table 5. Accuracy results of VEL & SOF

Parameters	Peak Area	Amount added(µg)	Amount recovered (µg)	% of recovery	% mean recovery
Velpatasvir					
50%	2807301	32.33	32.58	100.79	100.79
100%	5644767	64.66	65.52	101.33	101.33
150%	8332433	96.99	97.26	99.72	99.72
Sofosbuvir					
50%	276869	32.33	32.14	99.41	99.41
100%	5548876	64.66	64.41	99.61	99.61
150%	8506216	96.99	98.74	101.80	101.80

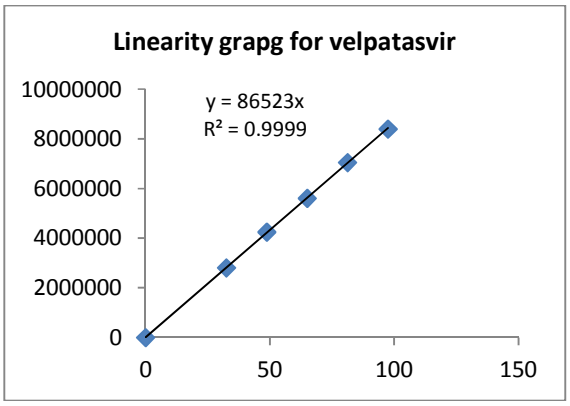

Fig. 6. Linearity curve of VEL

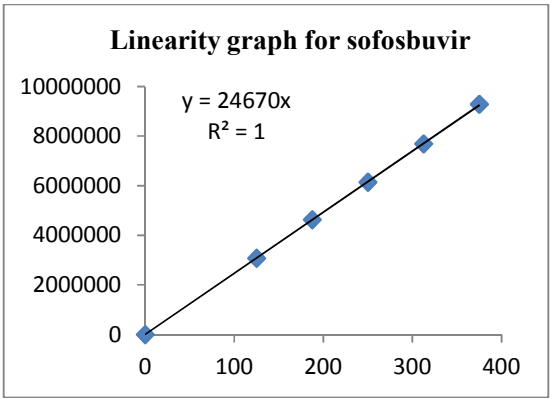

Fig. 7. Linearity curve of SOF

3.8 Robustness

The robustness of the method was unaffected when small, deliberate changes like, flow rate change, mobile phase composition, column temperature was performed and compared with normal conditions at 100% test concentration. The method was found to be robust for the said conditions. Results were tabulated in Table 6.

3.9 Analysis of Tablet Formulation

The proposed method was applied for the analysis of velpatasvir and sofosbuvir in tablet dosage forms, the results were found to be between 99.67-100.99%, the results summarized in Table 7.

3.10 Forced Degradation and Stability Indicating Studies

Non-interference of blank and degradants, the developed HPLC method proves the capability stability indicating a method for the analysis of VEL and SOF. Purity angle was less than the purity threshold and hence the proposed method was the specific and revealed its stability-indicating power. The results were summarized in Table 8. Fig. 8. (a-e) shows chromatograms of different stress degradation conditions.

Table 6. Results of robustness

S. No	Parameter	Condition	Velpatasvir			Sofosbuvir		
			RT	Peak Area	% Assay	RT	Peak Area	%Assay
1	Flow	0.8 ml/min	2.39	5476665	99.82	4.04	6410579	98.59
2		1 ml/min	2.85	5570645	101.53	4.79	6481226	99.67
3		1.2 ml/min	3.52	5526688	100.73	5.90	6564947	100.96
4	Temp	25 °C	2.84	5498542	100.22	4.76	6447497	99.16
5		30 °C	2.85	5570645	101.53	4.79	6481226	99.67
6		35 °C	2.85	5589293	100.87	4.80	6547497	101.47
7	Mobile Phase	B:M 78:19 v/v	2.68	5498542	100.22	4.22	6452436	99.23
8		B:M 78:22 v/v	2.85	5570645	101.53	4.79	6481226	99.67
9		B:M 78:25 v/v	2.86	5586765	101.83	5.26	6533379	100.48

Table 7. Assay results of VEL and SOF

Drug	Labelled amount mg/tab	Peak Area	% Assay
Velpatasvir	100	5570645	100.99
Sofosbuvir	400	6481226	99.67

Table 8. Degradation studies of VEL & SOF

Stress conditions	% Assay of the active moiety			
	Velpatasvir	% degradation	Sofosbuvir	% degradation
Acid (0.1 N HCl, refluxed for 1 H at 80ºC)	92.68	-7.32	92.06	-7.94
Base (0.1 N NaOH refluxed for 4H at 80ºC)	92.68	-7.32	92.98	-7.02
H_2O_2 (3% H_2O_2 Stored at room temperature for 2 H)	93.05	-6.95	92.71	-7.29
Water at for 6H 80^{o}C	89.69	-10.31	93.27	-6.73
UV light (near UV ≥200 for 10 days)	92.20	-7.80	92.81	-7.19

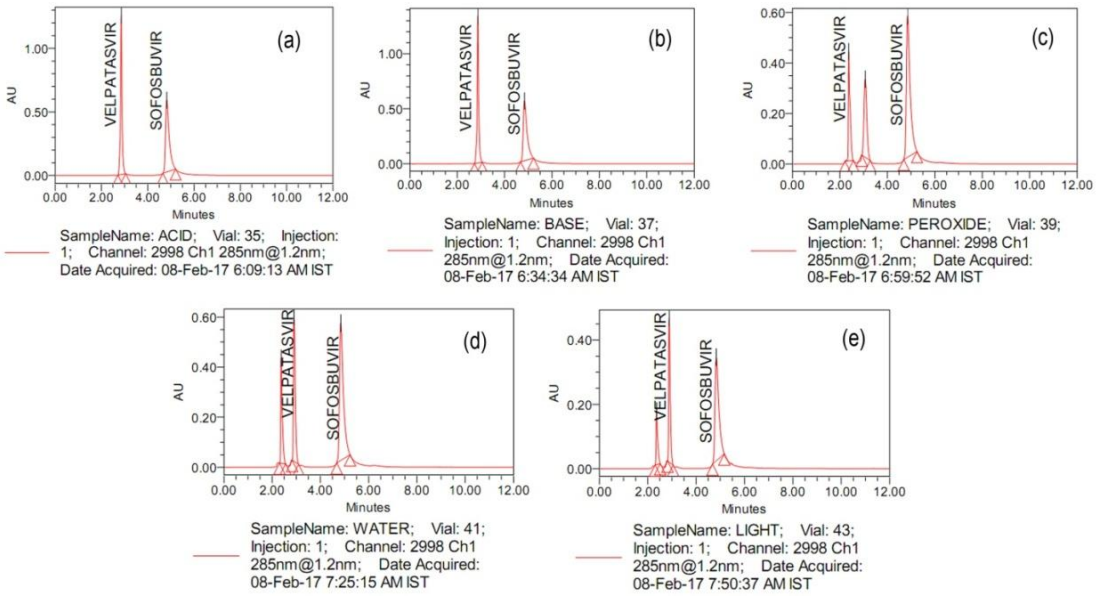

Fig. 8. Degradation Chromatogram working standard solutions of valpatasvir & sofosbuvir after (a) Acid hydrolysis (0.1 N HCl, refluxed for 1 H at 80ºC) (b) Alkali (0.1 N NaOH refluxed for 4H at 80ºC) and (c) Oxidative degradation (3% H_2O_2 Stored at room temperature) (d) Water degradation (e) UV light degradation (near UV ≥200 for 10 days)

SOF. Two drugs were subjected to various stress conditions like acid (0.1 N HCl, refluxed for 1 H at 80ºC), base (0.1 N NaOH refluxed for 4H at 80ºC), peroxide (3% H_2O_2 Stored at room temperature for 2H), water for 6H 80°C and light (near UV ≥200 for 10 days) stability studies were conducted on these samples. Hence the proposed method was the specific and revealed its stability-indicating power.

4. CONCLUSION

A simple, specific, precise and accurate isocratic HPLC-UV method was developed for the estimation of velpatasvir and Sofosbuvir in their pharmaceutical formulation. The two compounds were subjected to forced degradation applying several stress conditions. The proposed method was successfully separated two compounds with degradants, estimate the pharmaceutical active contents. The Proposed method was specific and stability-indicating power. Hence the developed method can be adapted to regular quality control analysis.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Shepard CW, Finelli L, Alter MJ, Global epidemiology of hepatitis C virus infection. Lancet Infect. Dis. 2005;5:558–567.
2. Lawitz E, Freilich B, Link J, et al. A phase 1, randomized, dose-ranging study of GS-5816, a once-daily NS5A inhibitor, in patients with genotype 1-4 hepatitis C virus. J Viral Hepat. 2015;22:1011–1019.
3. Ashis Mukhopadhya. Hepatitis C in India, Indian Academic of Sciences J. Biosci. 2008;33(4):465–473.
4. Mogalian E, German P, Kearney BP, et al. Use of multiple probes to assess transporter- and Cytochrome P450-mediated drug-drug interaction potential of the pangenotypic HCV NS5A inhibitor velpatasvir. Clin Pharmacokinetic. 2016; 55:605-613.
5. Younossi ZM, Stepanova M, Feld J, et al. Sofosbuvir/velpatasvir improves patient-reported outcomes in HCV patients: Results from ASTRAL-1 placebo-controlled trial. J Hepatol. 2016;65:33-39.

6. Feld JJ, Jacobson IM, Hézode C, et al. Sofosbuvir and Velpatasvir for HCV Genotype 1, 2, 4, 5, and 6 Infection. N Engl J Med. 2015;373:2599-2607.
7. Goodman LS, Gilman AG. The pharmacological basis of therapeutics, 9th Edn. By Hardman, J.G., Limbard, L.E., Editors in chief, McGraw – Hill, 1996.
8. Elkady, Ehab F, Aboelwafa, Ahmed A. A rapid and optimized LC-MS/MS Method for the Simultaneous extraction and determination of sofosbuvir and ledipasvir in human plasma. Journal of AOAC International. 2016;99(8):1252-1259.
9. Rezk MR, Basalious EB, Amin ME., Novel and sensitive UPLC-MS/MS method for quantification of sofosbuvir in human plasma: Application to a bioequivalence study. Biomed Chromatogram. 2016;30(9): 1354-1362.
10. Pan C, Chen Y, Chen W, Zhou G, Jin L, Zheng Y, Lin W, Pan Z., Simultaneous determination of ledipasvir, sofosbuvir and its metabolite in rat plasma by UPLC–MS/MS and its application to a pharmacokinetic study, Journal of Chromatography B. 2016;1008(1):255–259.
11. Ravikumar Vejendla CVS, Subramanyam, Veerabhadram G. Estimation and Validation of Sofosbuvir in Bulk and Tablet Dosage Form by RP-HPLC, Int J Pharm. 2016;6(2):121-127.
12. Mohan vikas , Satyanarayana T, Vinod Kumar D, Mounika E, Sri Latha M, Anusha R, Sathish Y., Development and valiation of new RP-HPLC method for the determination of Sofosbuvir in pure form. J Global Trends Pharm Sci. 2016;7(1):3013-3015.
13. Mohamed El-Kassem M Hassouna, Maha Mohammed Abdelrahman and Mahmoud Abdelfatah Mohamed., Assay and dissolution methods development and validation for simultaneous determination of Sofosbuvir and Ledipasvir by RP-HPLC Method in tablet dosage forms. J Forensic Sci & Criminal Inves. 2017;1(3):1-11.
14. Bakht Zaman, Faisal Siddique, Waseem Hassan., RP-HPLC Method for Simultaneous Determination of Sofosbuvir and Ledipasvir in tablet dosage form and its application to in vitro dissolution studies. Chromatographia. 2016;79(23):1605–1613.
15. Raj Kumar B, Subrahmanyam KV. A new validated RP-HPLC method for the simultaneous determination of simeprevir and sofosbuvir in pharmaceutical dosage form. Indo American Journal of Pharmaceutical Research. 2016;6(2):4508-4520.
16. Cristina del Barrio Gascon; Maria Buti., The Potential Role of Simeprevir for the Treatment of Hepatitis C. Future Virology. 2015;10 (2):67-75.
17. ICH-Q2(R1) Validation of Analytical Procedures: Text and Methodology International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland; 1996.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/40460