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Research Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF SOFOSBUVIR AND VELPATASVIR IN BULK AND PHARMACEUTICAL DOSAGE FORM

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A B S T R A C T

A simple, Accurate, precise method was developed for the simultaneous estimation of the Sofosbuvir and Velpatasvir in Tablet dosage form. Chromatogram was run through Std BDS 250 x 4.6 mm, 5µ. Mobile phase containing Buffer 0.1%OPA: Acetonitrile taken in the ratio 55:45 was pumped through column at a flow rate of 1 ml/min. Buffer used in this method was 0.1% OPA buffer. Temperature was maintained at 30°C. Optimized wavelength selected was 240.0 nm. Retention time of Sofosbuvir and Velpatasvir were found to 2.251 min and 2.633. %RSD of the Sofosbuvir and Velpatasvir were and found to be 0.4 and 0.4 respectively. %Recovery was obtained as 99.11% and 98.81% for Sofosbuvir and Velpatasvir respectively. LOD, LOQ values obtained from regression equations of Sofosbuvir and Velpatasvir were 0.16, 0.48 and 1.15, 3.47 respectively. Regression equation of Sofosbuvir is y = 1907.x+10654, y = 4963x+9760 of Velpatasvir. Retention times were decreased and run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

INTRODUCTION

The quality of a drug plays an important role in ensuring the safety and efficacy of the drugs. Quality assurance and control of pharmaceutical and chemical formulations is essential for ensuring the availability of safe and effective drug formulations to consumers. Hence Analysis of pure drug substances and their pharmaceutical dosage forms occupies a pivotal role in assessing the suitability to use in patients. The quality of the analytical data depends on the quality of the methods employed in generation of the data (1). Hence, development of rugged and robust analytical methods is very important for statutory certification of drugs and their formulations with the regulatory authorities.

The wide variety of challenges is encountered while developing the methods for different drugs depending on its nature and properties. This along with the importance of achieving the selectivity, speed, cost, simplicity, sensitivity, reproducibility and accuracy of results gives an opportunity for researchers to come out with solution to address the challenges in getting the new methods of analysis to be adopted by the pharmaceutical industry and chemical laboratories. Different physico-chemical methods (1) are used to study the physical phenomenon that occurs as a result of chemical reactions.

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Among the physico-chemical methods, the most important are optical (refractometry, polarimetry, emission and fluorescence methods of analysis), photometry (photocolorimetry and spectrophotometry covering UV-Visible, IR Spectroscopy and nepheloturbidimetry) and chromatographic (column, paper, thin layer, gas liquid and high performance liquid chromatography) methods. Methods such as nuclear magnetic resonance (NMR) and para magnetic resonance (PMR) are becoming more and more popular. The combination of mass spectroscopy (MS) with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures which are based on complex formation; acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have also been used in pharmaceutical analysis. The number of new drugs is constantly growing. This requires new methods for controlling their quality. Modern pharmaceutical analysis must need the following requirements.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of Pharmacopoeia.
3. The analysis should be economical.

The selected method should be precise and select The quality and safety of a drug is generally assured by monitoring and controlling the assay and impurities effectively. While assay determines the potency of the drug and impurities will determine the safety aspect of the drug. Assay of pharmaceutical products plays an important role in efficacy of the drug in patients.

MATERIALS AND METHODS

Materials

Sofosbuvir and Velpatasvir pure drugs (API), Combination Sofosbuvir and Velpatasvir tablets (epclusa), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem

Instruments

- Electronics Balance-Denver
- p^H meter -BVK enterprises, India
- Ultrasonicator-BVK enterprises
- WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector and Auto sampler integrated with Empower 2 Software.
- UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Sofosbuvir and Velpatasvir solutions.

Methods

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50

Preparation of Standard stock solutions: Accurately weighed 200 mg of Sofosbuvir, 50 mg of Velpatasvir and transferred to 50ml volumetric flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (4000µg/ml of Sofosbuvir and 1000µg/ml Velpatasvir)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (400µg/ml of Sofosbuvir and 100µg/ml of Velpatasvir)

Preparation of Sample stock solutions: 5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 100ml volumetric flask, 50ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (4000µg/ml of Sofosbuvir and 1000µg/ml of Velpatasvir)

Preparation of Sample working solutions (100% solution): 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (400µg/ml of Sofosbuvir and 100µg/ml of Velpatasvir)

Preparation of buffer

0.1%OPA Buffer*:* 1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water.

Validation

System suitability parameters

The system suitability parameters were determined by preparing standard solutions of Sofosbuvir (400ppm) and Velpatasvir (100ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined.

The % RSD for the area of six standard injections results should not be more than 2%.

Specificity: Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision

Preparation of Standard stock solutions: Accurately weighed 200 mg of Sofosbuvir, 50 mg of Velpatasvir and transferred to 50ml volumetric flasks and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (4000µg/ml of Sofosbuvir and 1000µg/ml Velpatasvir)

Preparation of Standard working solutions (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (400µg/ml of Sofosbuvir and 100µg/ml of Velpatasvir)

Linearity

25% Standard solution: 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (100µg/ml of Sofosbuvir and 25µg/ml of Velpatasvir)

50% Standard solution: 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (200µg/ml of Sofosbuvir and 50µg/ml of Velpatasvir)

75% Standard solution: 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (300µg/ml of Sofosbuvir and 75µg/ml of Velpatasvir)

100% Standard solution: 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (400µg/ml of Sofosbuvir and 100µg/ml of Velpatasvir)

125% Standard solution: 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (500µg/ml of Sofosbuvir and 125µg/ml of Velpatasvir)

150% Standard solution: 1.5ml each from two standard stock solutions was pipettede out and made up to 10ml (600µg/ml of Sofosbuvir and 150µg/ml of Velpatasvir)

Accuracy

Preparation of Standard stock solutions: Accurately weighed 200 mg of Sofosbuvir, 50 mg of Velpatasvir and transferred to 50ml volumetric flasks and $3/4^{th}$ of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (4000µg/ml of Sofosbuvir and 1000µg/ml Velpatasvir)

Preparation of 50% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Acceptance Criteria

The % Recovery for each level should be between 98.0 to 102

Robustness: Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. % RSD was within the limit.

LOD sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of Sofosbuvir, Velpatasvir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

LOQ sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each of Sofosbuvir, Velpatasvir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

Degradation studies

Oxidation

To 1 ml of stock solution of Sofosbuvir and Velpatasvir, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, the resultant solution was diluted to obtain 400µg/ml&100µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies

To 1 ml of stock s solution Sofosbuvir and Velpatasvir, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 400µg/ml&100µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies

To 1 ml of stock solution Sofosbuvir and Velpatasvir, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 400µg/ml& 100µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies

The standard drug solution was placed in oven at 105°C for 1 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 400µg/ml& 100µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Photo Stability studies

The photochemical stability of the drug was also studied by exposing the 4000µg/ml Sofosbuvir &1000µg/ml Velpatasvirµg/ml solution to UV Light by keeping the beaker in UV Chamber for 1days or 200 Watt hours/m^2 in photo stability chamber For HPLC study, the resultant solution was diluted to obtain 400µg/ml & 100µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 1hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to 400µg/ml& 100µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Assay of Sofosbuvir and Velpatasvir

Natco Pharma Limited, bearing the label claim Sofosbuvir 400mg, Velpatasvir 100mg. Assay was performed with the above formulation. Average % Assay for Sofosbuvir and Velpatasvir obtained was 99.64 and 99.58% respectively

RESULTS & DISCUSSIONS

Optimization of Chromatographic Conditions

To develop and establish a suitable RP-HPLC method for simultaneous estimation of Sofosbuvir and Velpatasvir, in bulk and Tablet dosage forms, different preliminary tests were performed and different chromatographic conditions were tested and optimized chromatographic conditions were developed.

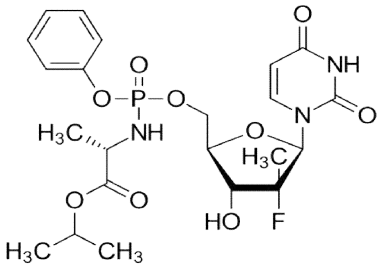

Figure 1 Chemical Structure of Sofosbuvir

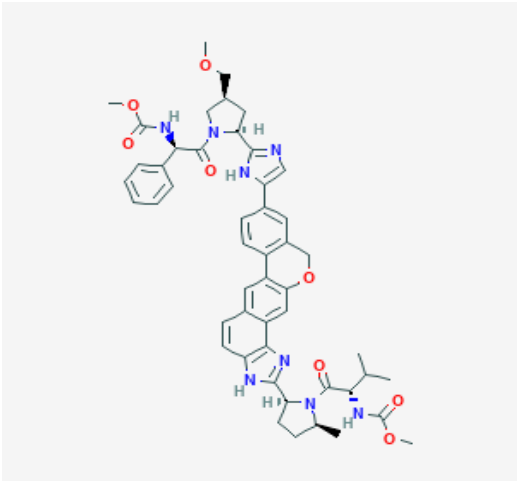

Figure 2 Chemical Structure of Velpatasvir

The final analysis was performed by using 50% Ortho phosphoric acid:45% Acetonitrile at a flow rate of 1ml/min. samples were analyzed at 260 nm detector wave length and at an injection volume of 10 µL using BDS C18 4.6 x 250mm, 5µm.with run time of 7 min. The proposed method was optimized to give sharp peak with good resolution and minimum tailing effect for Sofosbuvir and Velpatasvir,, the optimized chromatogram was obtained as shown in (Figure-3).

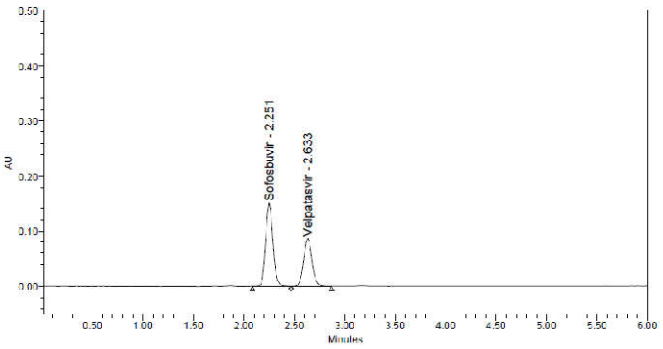

Figure 3 Optimised chromatogram of Sofosbuvir and Velpatasvir

Optimized method

Chromatographic conditions

Mobile phase : 55% OPA (0.1%): 45% Acetonitrile
Flow rate : 1 ml/min
Column : discovery C18 (4.6 x 250mm, 5µm)
Detector wave length : 260nm
Column temperature : 30°C
Injection volume : 10µL
Run time : 7 min
Diluent : Water and Acetonitrile in the ratio 50:50
Results : Both peaks have good resolution, tailing

Factor, theoretical plate count and resolution.

Validation

Linearity was established for Sofosbuvir (25-150µg/ml) and Velpatasvir, (12.5-75 µg/ml) at six different concentrations each were injected in a duplicates and average areas were determined and linearity equations were obtained as y = 43118.x + 27765 for Sofosbuvir and y = 31499.x + 35670 for Velpatasvir, Correlation coefficient (R^2) was determined as 0.999 for the two drugs. The Linearity calibration curves were plotted as shown in (Figure-4&5) for Sofosbuvir a and Velpatasvir, respectively.

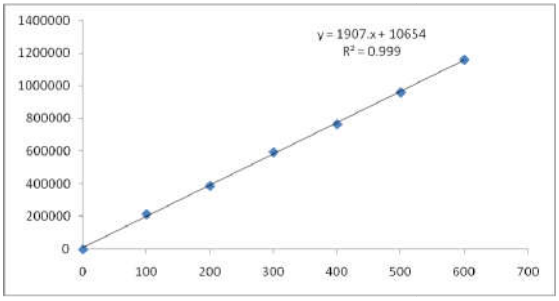

Fig No 4 Calibration curve of Sofosbuvir

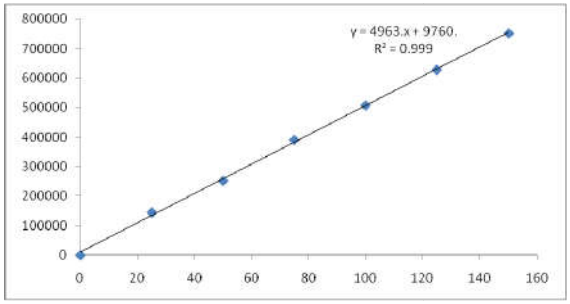

Fig No 5 Calibration curve of Velpatasvir

Table 1 Optimized Chromatographic Conditions

Parameter	Condition
RP-HPLC	WATERS HPLC SYSTEM equipped with quaternary pumps with PDA detector
Mobile phase	55% OPA (0.1%): 45% Acetonitrile
Flow rate	1ml/min
Column	discovery C18 (4.6 x 250mm, 5µm)
Detector wave length	260nm
Column temperature	30°C
Injection volume	10µL
Run time	7 min
Diluent	Water and Acetonitrile in the ratio 50:50
Retention Time	Sofosbuvir 2.251 min and Velpatasvir 2.633min
Theoretical Plates	Sofosbuvir 3950 and Velpatasvir 2946

Table 2 Accuracy table of Sofosbuvir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
	200	198.7425	99.37	
50%	200	198.4053	99.20	
	200	197.6649	98.83	
	400	394.7666	98.69	
100%	400	396.915	99.23	99.11%
	400	394.8526	98.71	
	600	598.0666	99.68	
150%	600	591.6382	98.61	
	600	597.8941	99.65	

Retention times of Sofosbuvir and Velpatasvir were 2.938min and 2.100 min respectively. Where no interfering peaks in blank and placebo at retention times of these drugs were not found in this method. So this method holds its specificity. Three levels of Accuracy samples 50%, 100%, 150% were prepared and Triplicates of injections were given for each level of accuracy and mean %Recovery was obtained as 99.68% and 99.34% for Sofosbuvir and Velpatasvir respectively.% RSD was calculated from the corresponding peaks obtained by injecting six times a known concentration of Sofosbuvir and Velpatasvir the repeatability was obtained as 0.5% and 0.6% respectively for Sofosbuvir and Velpatasvir and the % RSD for intermediate Precision was obtained as 1.0%, 1.3% for Sofosbuvir and Velpatasvir Low % RSD values indicates that the method developed was precise as shown in (Table-3).

Table 3 Accuracy table of Velpatasvir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
	50	49.50453	99.01	
50%	50	49.3200	98.64	
	50	49.57123	99.14	
	100	99.93452	99.93	
100%	100	98.65424	98.65	98.81%
	100	98.88918	98.89	
	150	147.3218	98.21	
150%	150	147.7296	98.49	
	150	147.5352	98.36	

The LOD and LOQ values were evaluated based on Relative standard deviation of response and slope of the calibration curve Sofosbuvir and Velpatasvir The detection limit values were obtained as 0.26 and 0.18 and Quantitation limit were fund to be 0.78 and 0.55 for Sofosbuvir and Velpatasvir Respectively as given in (Table-5).

Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus (65:35) mobile phase plus (55:45) temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner Table -5).

Table 4 Intermediate precision table of Sofosbuvir and Velpatasvir

S. No	Area of Sofosbuvir	Area of Velpatasvir
1.	763145	503110
2.	766043	504632
3.	767541	502354
4.	764097	500878
5.	762970	504451
6.	766596	503162
Mean	765065	503098
S.D	1920.6	1390.7
%RSD	0.3	0.3

Table 5 LOD and LOQ values of Sofosbuvir and Velpatasvir

Molecule	LOD	LOQ
Sofosbuvir	0.16	0.48
Velpatasvir	1.15	3.47

Table 6 Robustness Data of Sofosbuvir and Velpatasvir

S.no	Condition	%RSD of Sofosbuvir	%RSD of Velpatasvir
1	Flow rate (-) 1.1ml/min	0.1	0.3
2	Flow rate (+) 1.3ml/min	0.6	0.7
3	Mobile phase (-) 60B:40A	0.2	0.1
4	Mobile phase (+) 50B:50A	0.3	0.2
5	Temperature (-) 25°C	0.3	0.4
6	Temperature (+) 35°C	0.1	0.2

System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit. Sofosbuvir and Velpatasvir pure drugs (API) were obtained from spectrum Pharma research solutions and Asian Med Enterprises (Zepatir), bearing the label claim Sofosbuvir 10mg, Velpatasvir 5mg. Assay was performed with the above formulation. Average % Assay for Sofosbuvir and Velpatasvir obtained was 99.23% and 98.87% respectively the results were shown in (Table-7) and the chromatograms for Sofosbuvir and Velpatasvir standard drugs and pharmaceutical dosage forms were shown in fissgures Respectively.

Table 7 Assay Results of Sofosbuvir

S.no	Standard Area	Sample area	% Assay
1	765356	765567	99.67
2	764195	761880	99.19
3	763708	765430	99.65
4	769771	766049	99.73
5	760951	766956	99.85
6	766279	766334	99.77
Avg	765043	765369	99.64
Stdev	2940.3	1796.0	0.23
%RSD	0.4	0.2	0.23

Table 8 Assay Results of Velpatasvir

S.no	Standard Area	Sample area	% Assay
1	506936	504289	99.67
2	504866	501158	99.05
3	503283	505538	99.92
4	501637	505450	99.90
5	506381	501588	99.14
6	506559	505069	99.82
Avg	504944	503849	99.58
Stdev	2113.9	1972.4	0.39
%RSD	0.4	0.4	0.39

Table 9 Degradation Data of Sofosbuvir

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	4.94	0.206	0.344
2	Alkali	2.41	0.154	0.333
3	Oxidation	1.88	0.102	0.359
4	Thermal	0.93	0.164	0.348
5	UV	0.84	0.158	0.342
6	Water	0.84	0.172	0.352

Table 10 Degradation Data of Velpatasvir

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	4.64	0.476	0.612
2	Alkali	2.52	0.352	0.529
3	Oxidation	1.87	0.308	0.625
4	Thermal	0.04	0.164	0.348
5	UV	0.87	0.215	0.565
6	Water	0.92	0.285	0.567

Table 11 Summary Table

Parameters		Sofosbuvir	Velpatasvir	LIMIT
Linearity Range (µg/ml)		100-600 µg/ml	25-150µg/ml	
Regressioncoefficient		0.999	0.999	
Slope(m)		1907	4963	R< 1
Intercept(c)		10654	9760	
Regression equation (Y=mx+c)		y = 1907.x+10654	y = 4963x+9760	
Assay (% mean assay)		99.64%	99.58 %	90-110%
Specificity		Specific	Specific	No interference of any peak
System precision %RSD		0.4	0.4	NMT 2.0%
Method precision %RSD		0.2	0.4	NMT 2.0%
Accuracy %recovery		99.11%	98.81%	98-102%
LOD		0.16	1.15	NMT 3
LOQ		0.48	3.47	NMT 10
Robustness	FM	0.1	0.3	%RSD NMT 2.0
	FP	0.6	0.7	
	MM	0.2	0.1	
	MP	0.3	0.2	
	TM	0.3	0.4	
	TP	0.1	0.2	

Degradation Studies: Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation (Table -7&8).

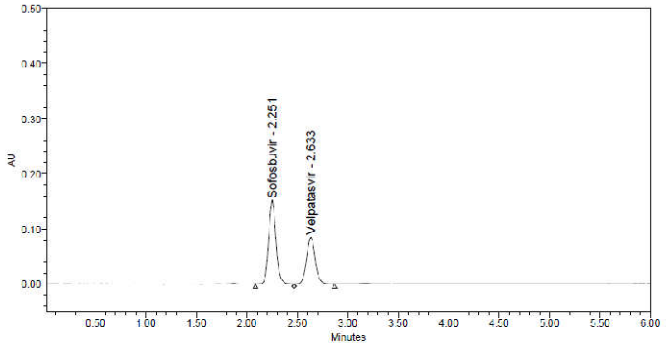

Figure 6 Standard Chromatogram of Sofosbuvir and Velpatasvir

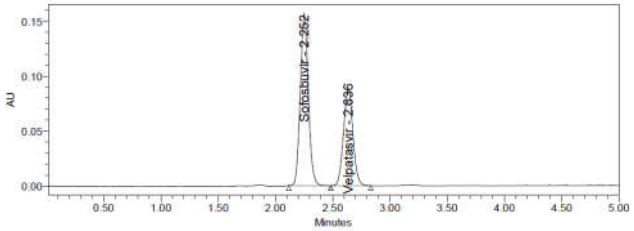

Fig 6.23 Inter Day precision Chromatogram

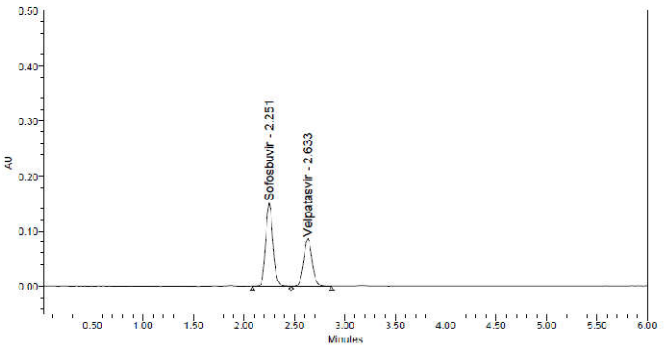

Figure 7 A Sample Chromatogram of Sofosbuvir and Velpatasvir in Pharmaceutical Dosage Form

CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Sofosbuvir and Velpatasvir in Tablet dosage form. Retention time of Sofosbuvir and Velpatasvir were found to be 2.251 min and 2.633. %RSD of the Sofosbuvir and Velpatasvir were and found to be 0.2 and 0.4 respectively. % Recovery was obtained as 99.11% and 98.81% for Sofosbuvir and Velpatasvir respectively. LOD, LOQ values obtained from regression equations of Sofosbuvir and Velpatasvir were 0.16, 0.48 and 1.15, 3.47 respectively. Regression equation of Sofosbuvir is y = 1907.x+10654, y = 4963x+9760 of Velpatasvir. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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