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

Sagar E. Tambe[1]*, Shivani S. Yendhe[2] and Sachin V. Datkhile[3]

[1]Department of Pharmaceutical Analysis and Quality Assurance, Samarth Institute of Pharmacy, Pune 412410
[2,3]Department of Pharmacology, Samarth Institute of Pharmacy 412410.

***Corresponding Author: Sagar E. Tambe**
Department of Pharmaceutical Analysis and Quality Assurance, Samarth Institute of Pharmacy, Pune 412410

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ABSTRACT
Objective: To develop UV-Visible spectrophotometric method for estimation of combination of Sofosbuvir and Velpatasvir. To develop analytical method for quantitation of combination of Sofosbuvir and Velpatasvir by RP-HPLC and Validate this method as per ICH guidelines.[1,2,3] **Method:** New Analytical method was developed for the estimation of Velpatasvir and Sofosbuvir by chromatographic separation. It was achieved on Grace C18 column (250mm* 4.6 ID, Particle size-5 micron) by employing a mobile phase comprised of Methanol:Water (80:20). The flow rate was 0.8ml/ minute and ultra violet detector was set at 266nm. The average retention time for Velpatasvir and Sofosbuvir was found to be 7.52 min and 4.72 min. **Results:** The method was optimized by using methanol and water (80:20) with flow rate 0.8 ml/min. The % assay was found to be 99.85%for sofosbuvir and 99.76% for Velpatasvir. The Robustness parameter i.e. standard deviation was found to be less than 2 for Sofosbuvir and Velpatasvir both. **Conclusion:** This novel method was found suitable for the routine quantitative analysis of Velpatasvir and Sofosbuvir in bulk and pharmaceutical dosage form. The method was accurate, precise, linear, reproducible, robust, and sensitive.

KEYWORDS: Sofosbuvir, Velpatasvir, Grace C18 column, Antiviral, Nucleotide Polymerase Inhibitor.

1. INTRODUCTION

Sofosbuvir[1,2,3,4,5]

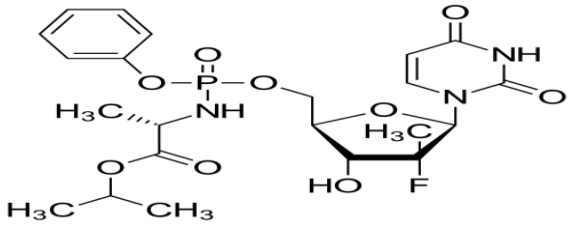

Fig. 1.1 Sofosbuvir

Application :Nucleotide polymerase inhibitor, treatment for hepatitis C
Purity : ≥98%
Molecular Weight : 529.458 g/mol
Molecular Formula : $C_{22}H_{29}FN_3O_9P$
Physical State : solid, white crystalline
Solubility : Soluble in Methanol
Storage : Store at room temperature below 300C
Melting Point : 120-125°C (lit.)
Pka : 9.3

Velpatasvir [1,2,3,4,5]

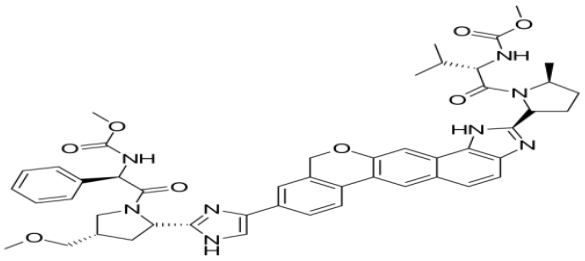

Fig. 1.2: Velpatasvir

Synonym: N-[(1R)-2-[(2S,4S)-2-[5-[1,11-dihydro-2-[(2S,5S)-1-[(2S)-2-[(methoxycarbonyl)amino]-3-methyl-1-oxobutyl]-5-methyl-2-pyrrolidinyl][2]benzopyrano[4',3':6,7]naphth[1,2-d]imidazol-9-yl]-1H-imidazol-2-yl]-4-(methoxymethyl)-1-pyrrolidinyl]-2-oxo-1-phenylethyl]-carbamic acid, methyl ester.
Application: Velpatasvir is a Direct-Acting Antiviral medication to treat
Chronic Hepatitis C.
Purity: ≥98%
Molecular Weight : 883.02 g/mol

Molecular Formula	:	$C_{49}H_{54}N_8O_8$
Physical State	:	crystalline Solid
Solubility	:	Soluble in organic solvents such as ethanol, Dimethyl Formamide.
Storage	:-20° C	
Melting Point	:	170-174 °C (lit.)
pKa	:	9.3 (Predicted)

2. MATERIALS AND METHODS

Materials:

Sofosbuvir, Velpatasvir, Combination Sofosbuvir and Velpatasvir tablets, distilled water, methanol etc.[6,7,8,12,14]

Instrument:

1. HPLC instrument used was of HPLC Binary Gradient System, model no. HPLC 3000 series with Auto Injector.
2. UV-VIS spectrophotometer UV-3000-M.
3. Sonicator (Wenser Ultra Sonicator)
4. P^H meter (Thermo scientific)
5. Micro balance (Sartorius)
6. Vacuum filter pump

Reagents used: Methanol HPLC Grade, Distilled Water[6,7,8]

Methods:

Standard preparation:

Accurately weighed and transferred 10mg of Sofosbuvir and 10mg of Velpatasvir working Standards into a 10ml clean dry volumetric flasks, added 3/4th volume of diluent, sonicated for 5 minutes and made up to the final volume with diluent. From the above stock solution 0.2ml,0.4ml, 0.6ml, 0.8ml, 1ml of Sofosbuvir and 0.05ml, 0.1ml, 0.15ml, 0.2ml, 0.25ml of Velpatasvir were pipetted into a 10ml volumetric flask and made up to 10ml with diluent to get a mixed standard solution containing concentration of 20, 40, 60, 80, 100ppm of Sofosbuvir and 5, 10, 15, 20, 25ppm of Velpatasvir respectively.[6,7,8,12,14]

Sample preparation:

Weighed accurately combined twenty tablets of sofosbuvir and velpatasvir and prepare fine powder. A quantity of tablet powder equivalent to 10mg of Sofosbuvir and 2.5mg of Velpatasvir (16.12mg) was accurately weighed and transferred into a 10ml volumetric flask. 5 ml of mobile phase (methanol:water, 80:20) was added to the volumetric flask and ultrasonicated for 25 min; the volume was make up to the 10ml and mixed well. From that pipette out 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1ml solution and dissolve in 10ml of mobile phase. These solutions having concentrations of 20, 40, 60, 80, 100ppm of sofosbuvir and 5, 10, 15, 20, 25ppm of velpatasvir.[6,7,8,12,14]

3. METHOD DEVELOPMENT

Optimized method in combination: Sofosbuvir and Velpatasvir drugs were eluted with good retention time, resolution, all the system suitable parameters like Plate count and Tailing factor were within the limits.[9,10,11,12,14]

Column Used : Grace C18 (250mm* 4.6 ID, Particle size-5 micron)
Mobile phase : Methanol: Water (80:20)
Flow rate : 0.8ml/min
Wavelength : 260nm
Temperature : 30°C
Injection volume : 10µl
Retention time of sofosbuvir: 4.720min
Retention time of velpatasvir: 7.520min
Run time : 10.93 min

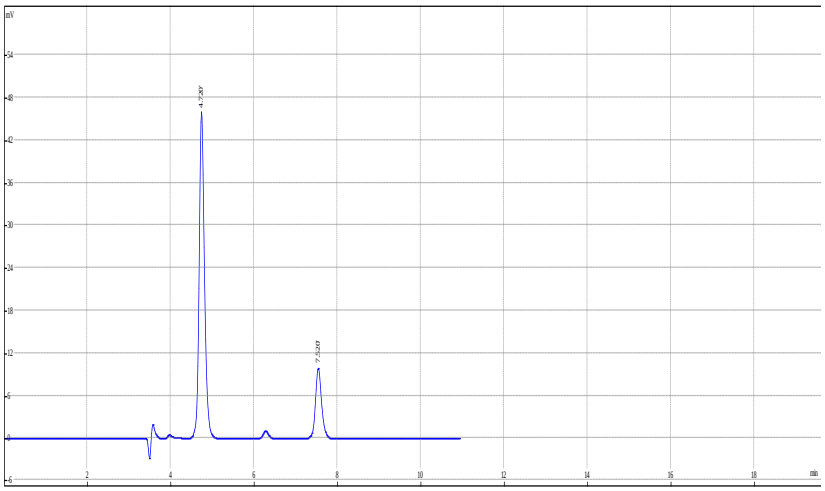

Fig 3.1: Trial 3 Optimized combined chromatogram of Sofosbuvir and Velpatasvir.

Assay:

Standard preparations are made from the API and Sample Preparations are from Formulation. By taking 60ppm of sofosbuvir and 15ppmof velpatasvir for both sample and standard. Both sample and standards are injected. Drug in the formulation was estimated by taking the standard as the reference. The Average %Assay was calculated and found to be 99.85% for Sofosbuvir and 99.76% for Velpatasvir respectively.[9,10,11,12,14]

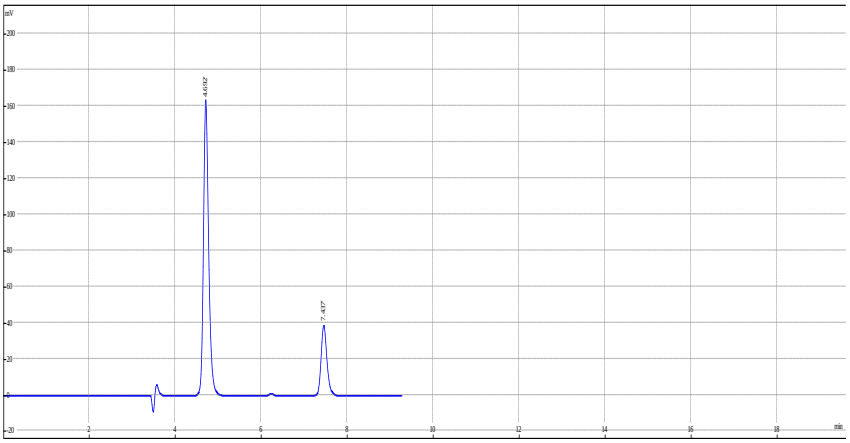

Fig. 3.2: Assay of tablet chromatogram of Sofosbuvir and Velpatasvir.

Table 3.1: Assay of tablet.

S. No.	Drug	Area of standard	Area of sample	%Assay
1	Sofosbuvir	1988529	1985620	99.85%
2	Velpatasvir	626126	624665	99.76%

4. METHOD VALIDATION

1. **System suitability:** All the system suitability parameters are within range and satisfactory as per ICH guidelines. [1,5,7,12,14]

System suitability parameters

The system suitability parameters were determined by preparing standard solutions of Sofosbuvir and Velpatasvir and the solutions were injected six times and the parameters like peak tailing, resolution and Theoretical plate count were determined.

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

Table 4.1: System suitability parameters.

Property	Sofosbuvir	Velpatasvir
Resolution	Greater than 1.75	Greater than 1.75
Theoretical plates(N)	Greater than 2000	Greater than 2000
Tailingfactor (T)	Less than 2	Less than 2

2. **Accuracy:**

Accuracy of analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometime termed trueness. Accuracy should be established across the specified range of the analytical procedure.[1,5,7,12,14]

Table 4.2: Sofosbuvir accuracy table.

		Standard Deviation		Accuracy	Precision
Conc.	Area	Mean	SD	%SD	%RSD
	523876				
20	522737	523941.6667	1238.806011	0.23643968	0.236439682
	525212				
	1988529				
60	1986193	1988035	1651.378818	0.08306588	0.083065883
	1989383				
	3483561				
100	3485756	3485733.667	2161.586531	0.06201238	0.062012384
	3487884				

Table 4.3: Velpatasvir accuracy table.

		Standard Deviation		Accuracy	Precision
Conc.	Area	Mean	SD	%SD	%RSD
	165050				
5	165127	165116.6667	62.14767362	0.03763864	0.037638644

	165173				
	626126				
15	627541	626346	1101.601107	0.17587741	0.175877408
	625371				
	1069609				
25	1067069	1069150.333	1894.118616	0.17716111	0.177161112
	1070773				

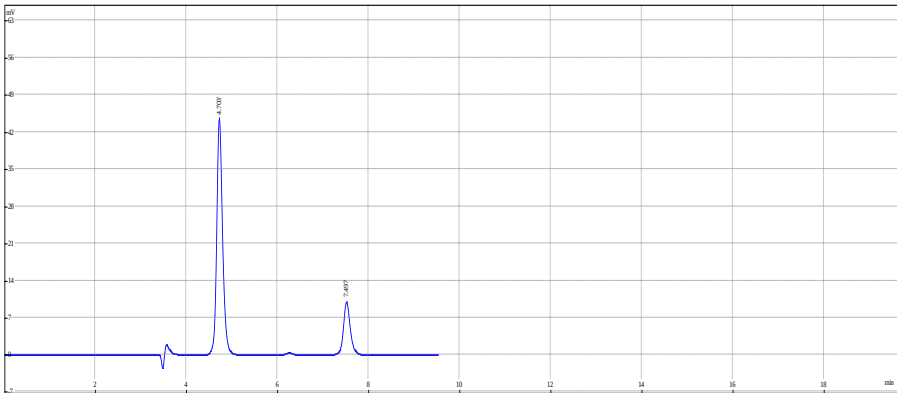

Fig. 4.1: Sofosbuvir 20ppm and Velpatasvir 5ppm Accuracy.

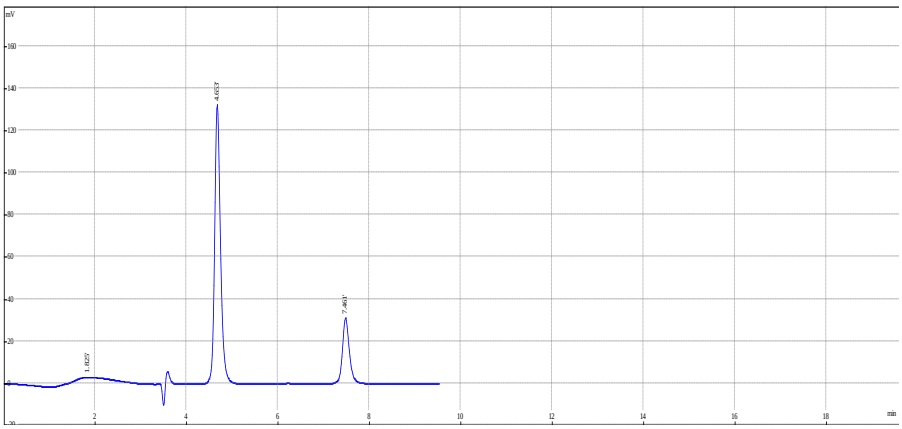

Fig. 4.2: Sofosbuvir 60ppm and Velpatasvir 15ppm Accuracy.

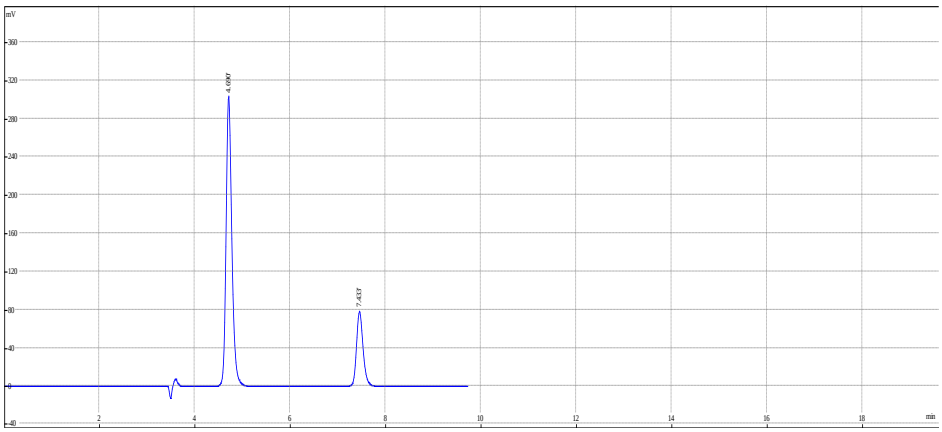

Fig. 4.3: Sofosbuvir 100ppm and Velpatasvir 25ppm Accuracy.

Percentage recovery:
The % Recovery for each level should be between 98.0 to 102.

50%: Weighed accurately combined twenty tablets of sofosbuvir and velpatasvir and prepare fine powder. A quantity of tablet powder equivalent to 10mg of Sofosbuvir and 2.5mg of Velpatasvir (16.12mg) was accurately weighed and transferred into a 10ml volumetric flask. 5 ml of mobile phase (methanol:water,80:20) was added to the volumetric flask and ultrasonicated for 25 min; the volume was

make up to the 10ml and mixed well. From that pipette out 0.2ml solution and dissolve in 10ml of mobile phase. These solutions having concentrations of 20ppm sofosbuvir and 5ppm of velpatasvir.[1,5,7,12,14]

100%: Weighed accurately combined twenty tablets of sofosbuvir and velpatasvir and prepare fine powder. A quantity of tablet powder equivalent to 10mg of Sofosbuvir and 2.5mg of Velpatasvir (16.12mg) was accurately weighed and transferred into a 10ml volumetric flask. 5 ml of mobile phase (methanol:water,80:20) was added to the volumetric flask and ultrasonicated for 25 min; the volume was make up to the 10ml and mixed well. From that pipette out 0.4ml solution and dissolve in 10ml of mobile phase. These solutions having concentrations of 40ppm sofosbuvir and 10ppm of velpatasvir.[1,5,7,12,14]

150%: Weighed accurately combined twenty tablets of sofosbuvir and velpatasvir and prepare fine powder. A quantity of tablet powder equivalent to 10mg of Sofosbuvir and 2.5mg of Velpatasvir (16.12mg) was accurately weighed and transferred into a 10ml volumetric flask. 5 ml of mobile phase (methanol:water,80:20) was added to the volumetric flask and ultrasonicated for 25 min; the volume was make up to the 10ml and mixed well. From that pipette out 0.6ml solution and dissolve in 10ml of mobile phase. These solutions having concentrations of 60ppm sofosbuvir and 15ppm of velpatasvir. [1,5,7,12,14]

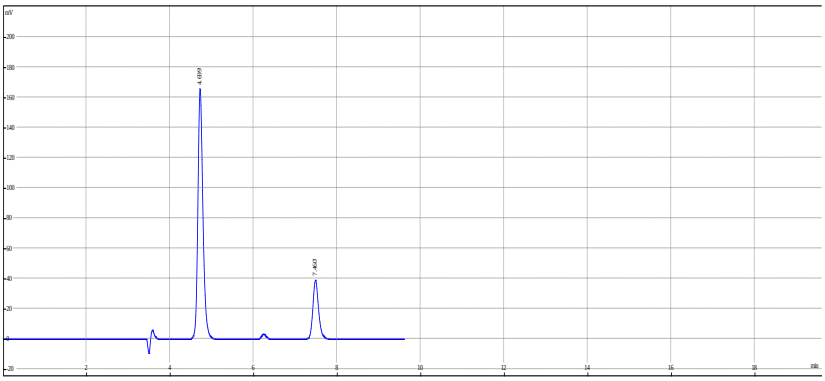

Fig. 4.4: 50% Recovery of Sofosbuvir and Velpatasvir.

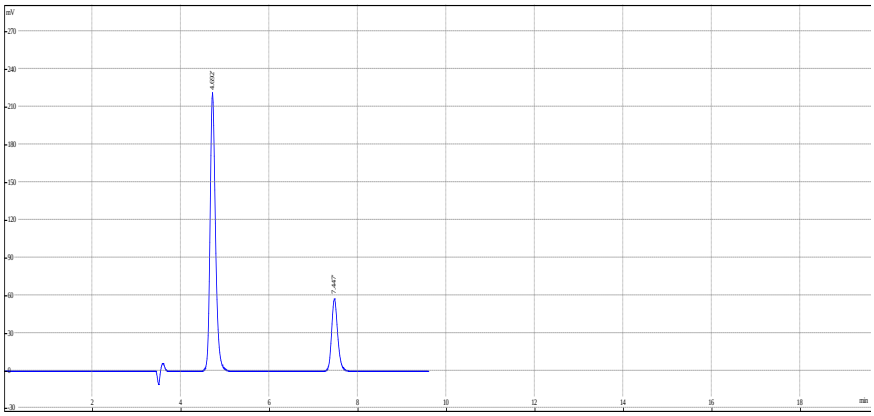

Fig. 4.5: 100% Recovery of Sofosbuvir and Velpatasvir.

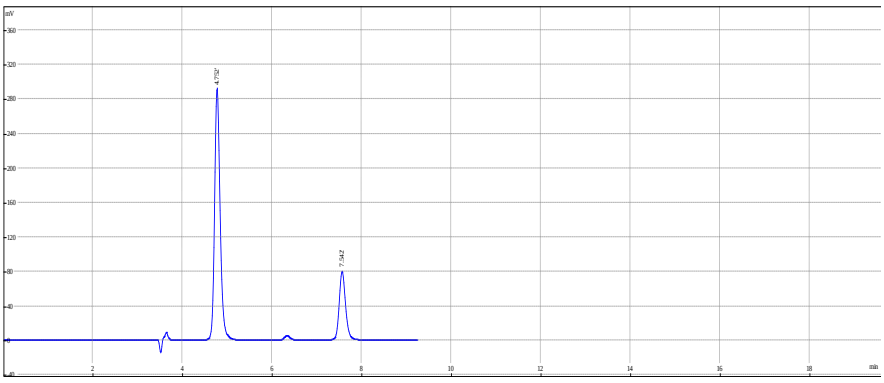

Fig. 4.6: 150% Recovery of Sofosbuvir and Velpatasvir.

Table 4.4: Sofosbuvir % recovery.

Sr. NO.	% Composition	Area of Standard	Area of Sample	% Recovery
1	**50% Recovery**	1988529	1982674	99.70556125
2	**100% Recovery**	2790410	2789000	99.94946979
3	**150% Recovery**	3483561	3477603	99.82896812

Table 4.5: Velpatasvir % recovery.

Sr. NO.	% Composition	Area of Standard	Area of Sample	% Recovery
1	50% Recovery	626126	622197	99.37249052
2	100% Recovery	879866	878222	99.81315337
3	150% Recovery	1069609	1069455	99.98560222

3. Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between the series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. [12,14]

Acceptance criteria:

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

Sample preparation:

Weighed accurately combined ten tablets of sofosbuvir and velpatasvir and prepare fine powder. A quantity of tablet powder equivalent to 10mg of Sofosbuvir and 2.5mg of Velpatasvir (16.12mg) was accurately weighed and transferred into a 10ml volumetric flask. 5 ml of mobile phase (methanol:water, 80: 20) was added to the volumetric flask and ultrasonicated for 25 min; the volume was make up to the 10ml and mixed well. From that required concentrations were taken.[12,14]

Intraday precision (Repeatability):

Intraday Precision was performed and % RSD for Sofosbuvir and Velpatasvir were found to be 0.08% and 0.28% respectively.[12,14]

Table: 4.6: Intraday Precision results for Sofosbuvir and Velpatasvir.

Sr. No.	Sofosbuvir	Velpatasvir
1	1988529	626126
2	1986193	627541
3	1989383	626687
4	1985742	624079
5	1986222	624135
6	1985750	628457
Mean	1986970	626170.8
%RSD	0.08%	0.28%

Interday precision: Inter day precision was performed with 24 hrs time lag and the %RSD Obtained for Sofosbuvir and Velpatasvir were 0.11% and 0.21%.[12,14]

Table: 4.7: Interday precision results for Sofosbuvir and Velpatasvir.

Sr. No.	Sofosbuvir	Velpatasvir
1	1988529	626126
2	1986193	627541
3	1989383	626687
4	1984383	628152
5	1989310	626976
6	1985186	628842
Mean	1985186	628842
%RSD	0.11%	0.21%

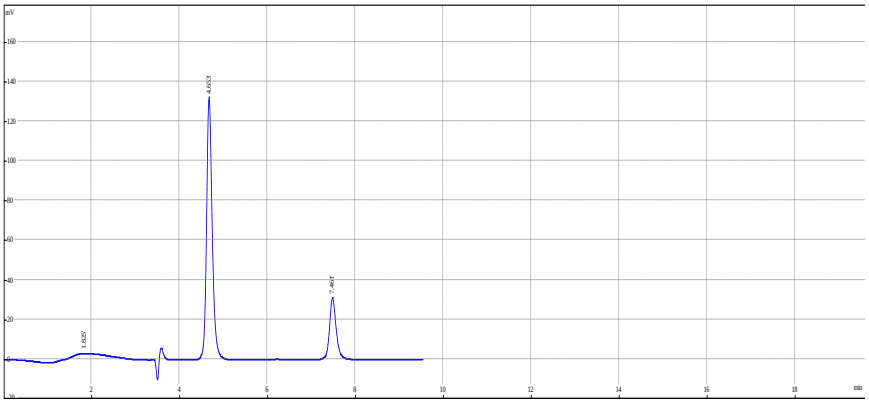

Fig. 4.7: Intraday morning Sofosbuvir 60ppm and Velpatasvir 15ppm.

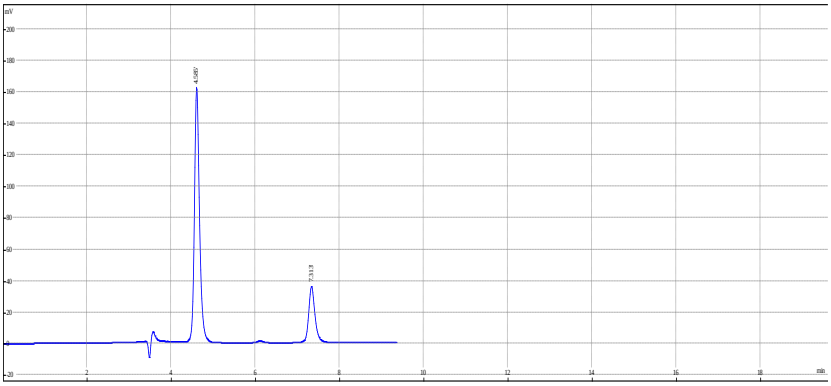

Fig. 4.8: Intraday evening Sofosbuvir 60ppm and Velpatasvir 15ppm.

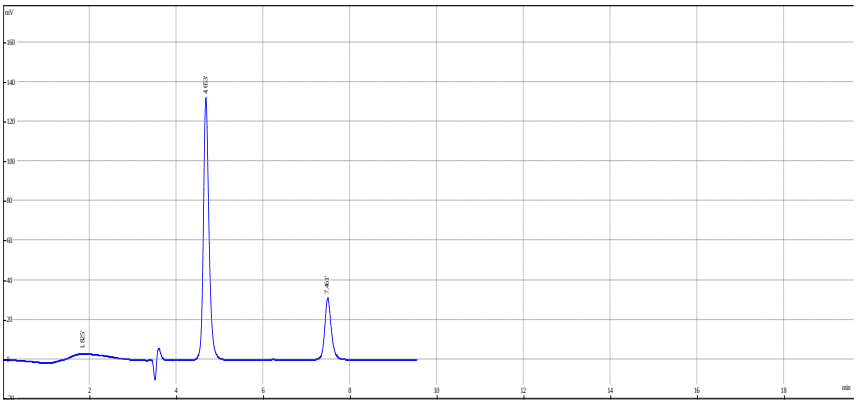

Fig. 4.9: Interday day 1 Sofosbuvir 60ppm and Velpatasvir 15ppm.

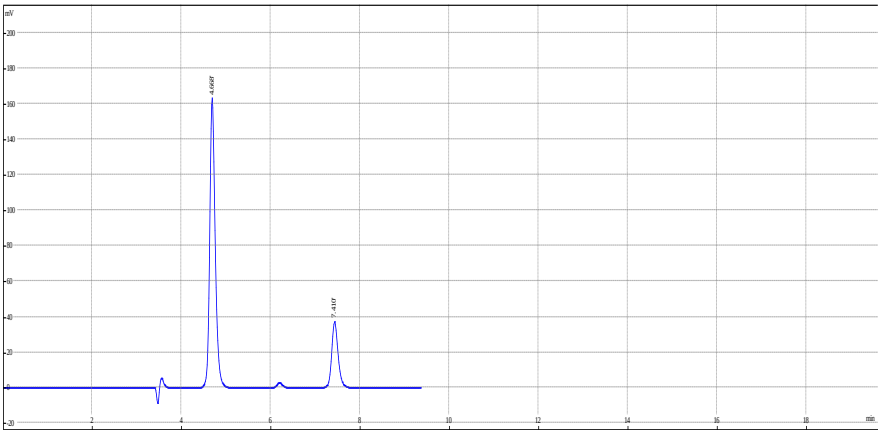

Fig. 4.10: Interday day 2 Sofosbuvir 60ppm and Velpatasvir 15ppm.

4. Linearity:

The linearity of an analytical procedureis stability(within given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. [12,14]

Table 4.8: Linearity for Sofosbuvir and Velpatasvir (Conc. Vs Area).

Conc.	Area of Sofosbuvir	Conc.	Area of Velpatasvir
20	523876	5	165050
40	1296786	10	401492
60	1988529	15	626126
80	2790410	20	879866
100	3483561	25	1069609

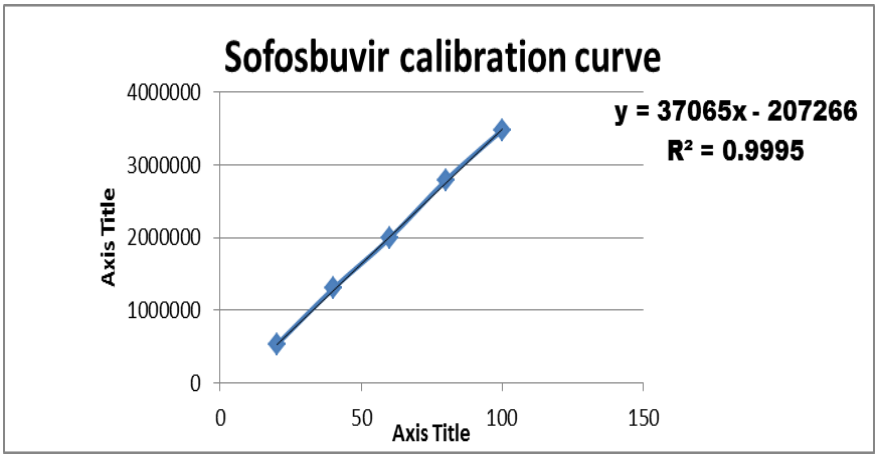

Fig. 4.11: Calibration curve of sofosbuvir.

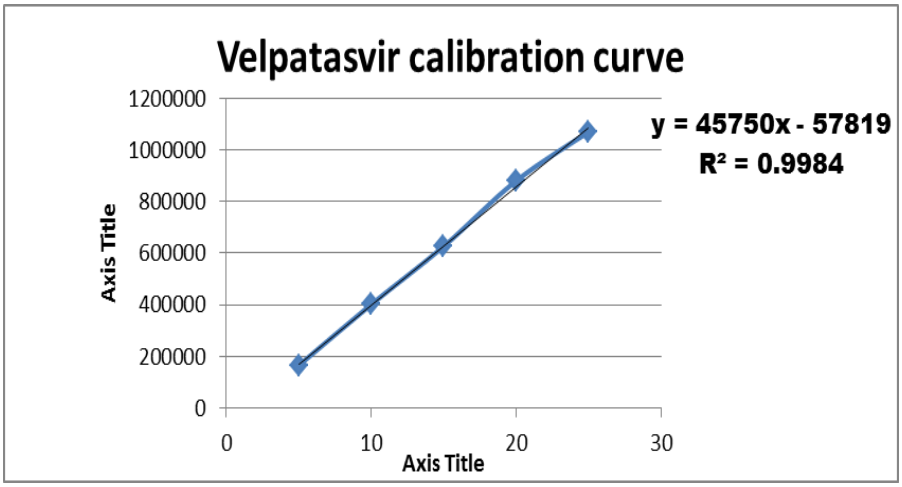

Fig. 4.12: Calibration curve of Velpatasvir.

Acceptance criteria

Correlation co efficient(R^2) should be 0.999. Five Linear concentrations of Sofosbuvir (20ppm to 100ppm) and Velpatasvir (5ppm to 25ppm) are prepare and Injected. Regression equation of the Sofosbuvir and Velpatasvir.

5. Limit of detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated under stated experimental conditions.

$$LOD = 3.3 * standard\ deviation\ of Intercept / Average\ of\ Slope$$

Limit of detection was calculated by intercept method and LOD for Sofosbuvir and Velpatasvir were found to be 0.0143 and 0.0711 respectively. Standard deviation calculated from accuracy by taking mean of three readings. Slope calculated from linearity by using formula, Slope = Y2-Y1/X2-X1. [12,14]

6. Limit of quantitation

The quantitation limit of an individual analytical procedure is defined as the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

$$LOQ = 10 * standard\ deviation\ of Intercept / Average\ of\ Slope$$

Limit of Quantification was calculated by intercept method and LOQ for Sofosbuvir and Velpatasvir were found to be 0.04357 and 0.2155 respectively. Standard deviation calculated from accuracy by taking mean of three readings. Slope calculated from linearity by using formula, Slope = Y2-Y1/X2-X1. [12,14]

7. Robustness:

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

Acceptance criteria: RSD <2%

Robustness conditions like mobile phase minus, mobile phase plus, pH minus and pH plus was maintained and samples were injected in duplicate manner. System suitability parameters were not much effected and all the parameters were passed. %RSD was within the limit.[12,14]

Table 4.9: Robustness data of sofosbuvir.

		Conc.	Area	Mean	SD	%SD
Mobile Phase	75:25	40	1294138	1295981	1600.01	0.123459%
	85:15	40	1297018			
	80:20	40	1296786			
pH	2.4	40	1293659	1295560	1669	0.128824%
	3.6	40	1296234			
	3.0	40	1296786			

Table 4.10: Robustness data of velpatasvir.

		Conc.	Area	Mean	SD	%SD
Mobile Phase	75:25	10	404661	402567	1813.67	0.450527%
	85:15	10	401548			
	80:20	10	401492			
pH	2.4	10	401766	402249	1082.58	0.269130%
	3.6	10	403489			
	3.0	10	401492			

8. Ruggedness: Ruggedness is defined as the degree of reproducibility of the test results obtainedunder a variety of normal test condition, such as different laboratories, different analyst, different instruments, different lots of reagents, different elapsed assay times, different temp, different days etc.[12,14]

Table 4.11 Ruggedness data of Sofosbuvir and Velpatasvir.

Sofosbuvir		Velpatasvir	
Conc.	Area	Conc.	Area
20	530186	5	167737
40	1309763	10	404943
60	1999282	15	629381
80	2796792	20	886412
100	3486230	25	1075044

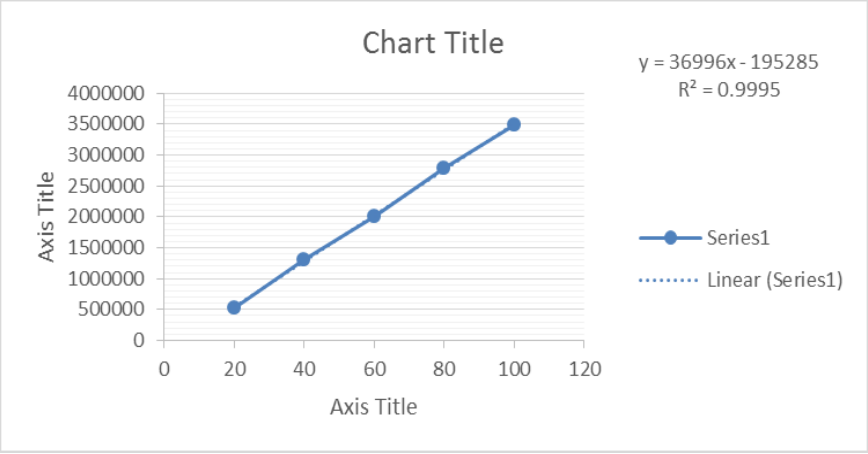

Fig. 4.13: Ruggedness curve of sofosbuvir.

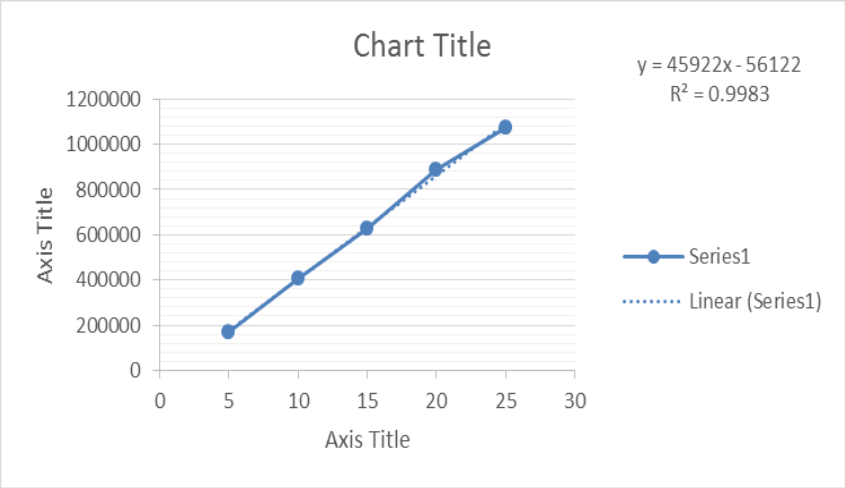

Fig. 4.14: Ruggedness curve of velpatasvir.

5. RESULTS

For the method development Sofosbuvir and Velpatasvir lot of trails was performed and finally we optimized the method by using methanol and water (80:20) the flow rate was 0.8 ml/min. Then Grace C18 (250mm * 4.6 ID, Particle size: 5 micron) by used as stationary phase. The detection wave length was 260 nm. The temperature ambient inject volume 10 micro meter. The developed method was further goes for method validation as per ICH guidelines Tthe accuracy parameter result was formed to be for Sofosbuvir and Velpatasvir standard deviation less than 2%. The repeatability parameter result was formed to be for Sofosbuvir this much 0.08% and from Velpatasvir 0.28%. The intermediate precision parameter result was formed to be for Sofosbuvir this much 0.11% and from Velpatasvir 0.21%. The detection limit parameter result was formed to be for Sofosbuvir this much 0.014ppm and from Velpatasvir 0.071 ppm. The quantization limit parameter result was formed to be for Sofosbuvir this much 0.0.043ppm and from Velpatasvir 0.215 ppm. The linearity parameter (R^2)result was formed to be for Sofosbuvir this much 0.999 and from Velpatasvir 0.998. The Robustness parameter i.e. standard deviation was found to be less than 2 for Sofosbuvir and Velpatasvir both.[12,13,14]

Last the all results of performed validation was found in the limit.

Table 5.1: Summary table.

Parameters	Acceptance Criteria	Sofosbuvir	Velpatasvir
Accuracy/trueness	SD<2% (individual)	<2%	<2%
% Recovery	50%	99.70%	99.37%
	100%	99.94%	99.81%
	150%	99.82%	99.98%
Repeatability	RSD <2%	0.08%	0.28%
IntermediatePrecision	RSD <2%	0.11%	0.21%
DetectionLimit		0.014ppm	0.071ppm
QuantizationLimit		0.043 ppm	0.215ppm
Linearity	R^2 is near to 1	0.999	0.998
Robustness	SD<2%	SD<2%	SD<2%
Ruggedness	R^2 is near to 1	0.999	0.998

6. CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Sofosbuvir and Velpatasvir in Tablet dosage form. Retention times of Sofosbuvir and Velpatasvir were found to be 4.72 min and 7.52 min. For the method development Sofosbuvir and Velpatasvir lot of trails was performed and finally we optimized the method by using methanol and water (80: 20) the flow rate was 0.8 ml/min. Then Grace C18 (250mm * 4.6 ID, Particle size: 5 micron) by used as stationary phase. The detection wave length was 260 nm. The temperature ambient inject volume 10 micro meter. The developed method was further goes for method validation as per ICH guidelines .the accuracy parameter result was formed to be for Sofosbuvir and Velpatasvir standard deviation less than 2%. The repeatability parameter result was formed to be for Sofosbuvir this much 0.08% and from Velpatasvir 0.28%. The intermediate precision parameter result was formed to be for Sofosbuvir this much 0.11% and from Velpatasvir 0.21%. The detection limit parameter result was formed to be for Sofosbuvir this much 0.014ppm and from Velpatasvir 0.071 ppm. The quantization limit parameter result was formed to be for Sofosbuvir this much 0.0.043ppm and from Velpatasvir 0.215 ppm. The linearity parameter (R^2) result was formed to be for

Sofosbuvir this much 0.999 and from Velpatasvir 0.998. The Robustness parameter i.e. standard deviation was found to be less than 2 for Sofosbuvir and Velpatasvir both. Retention times are 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.[12,13,14]

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