# DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-UPLC METHOD FOR THE ESTIMATION OF SOFOSBUVIR AND VELPATASVIR IN PHARMACEUTICAL DOSAGE FORM.

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## Abstract

A novel approach was used to develop a precise, accurate, simple and repeatable RP-UPLC method for the simultaneous estimation of Sofosbuvir and Velpatasvir in tablet dosage form. The formulation was determined by using Aquity SB $C_{18}$ column (100 x 2 mm, 1.8µ) as stationary phase and phosphate buffer: Acetonitrile was used as mobile phase. The PH was adjusted to 2.8 by using triethyl amine and the flow rate was maintained at 0.2 ml/min. Quantification was achieved of sofosbuvir and Velpatasvir at 250 nm with PDA detector.The retention time for sofosbuvir and Velpatasvir was found to be 1.472 and 1.836 minute respectively. The linearity for sofosbuvir and Velpatasvir was obtained in the concentration range of 100-600 µg/ml and 25-150 µg/ml with mean accuracies of 98.30% and 99.08% respectively. The method was successfully applied to pharmaceutical formulation because no chromatographic interferences from tablet excipients were found. The precision (intraday, interday and repeatability) of method was found within limits. The method was validated as per ICH guidelines. Sofosbuvir and Velpatasvir API and market formulation were subjected to acid and alkali hydrolysis, oxidation, thermal and photolytic forced degradation. The degraded product peaks were well resolved from the pure drug peak with significant difference in their retention time values. Besides, the peak purity of drug substance and drug product peak also confirmed the specificity of the methods with respect to the degradation products. In the forced degradation study Sofosbuvir and Velpatasvir showed maximum degradation in acid stress study followed by less degradation in oxidative stress condition. The developed isocratic method was simple, specific, sensitive, and economic and can be used for estimation of . Sofosbuvir and Velpatasvir in bulk and their combined tablet dosage form for routine analysis and stabilitystudies.

***Key words:*** RP- UPLC, Sofosbuvir, velpatasvir, isocratic.

## 1 INTRODUCTION

Sofosbuvir (SOF) (Fig.1) is a medication used for the treatment of hepatitis C. It is only recommended with some combination of ribavirin, peginterferon-alfa, simeprevir, ledipasvir, or daclatasvir. Cure rates are 30 to 97% depending on the type of hepatitis C virus involved. Safety during pregnancy is unclear; while, some of the medications used in combination may result in harm to the baby. It is taken orally. The IUPAC name of SOF is Isopropyl (2S)-2-[[[(2R,3R,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl- tetrahydrofuran-2-yl] methoxy-phenoxy-phosphoryl] amino] propanoate, Molecular formula $C_{22}H_{29}FN_3O_9P$, Molecular weight 529.4 g/mol. Literature survey indicate that it had been validated by HPLC, and by UV spectrophotometry.

Velpatasvir (VEL) is an NS5A inhibitor which is used together with sofosbuvir in the treatment of hepatitis C infection of all six major genotypes. Velpatasvir (Fig.2) is chemically Methyl {(2S ) - 1 -[ ( 2S, 5S) – 2 - (9 - { 2 -[(2S ,4S) – 1 - {(2R) - 2 -[(methoxycarbonyl) amino]-2 - phenylacetyl} - 4- (methoxymethyl)-2 -pyrrolidinyl] -1 H - 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} carbamate used as an anti-cholinergic and anti- spasmodic. Molecular formula $C_{49}H_{54}N_8O_8$, Molecular weight 883.02 g/mol.

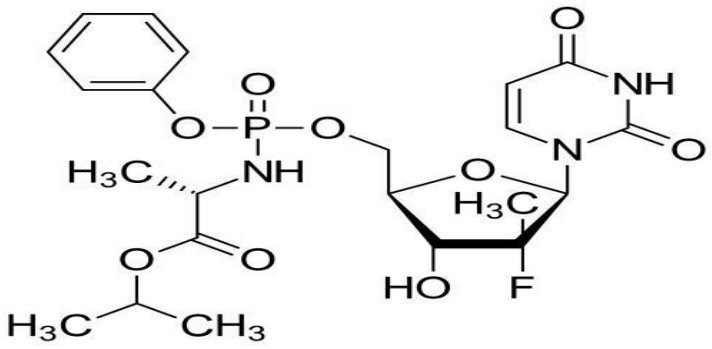


**Figure 1: Chemical Structure of Sofosbuvir.**

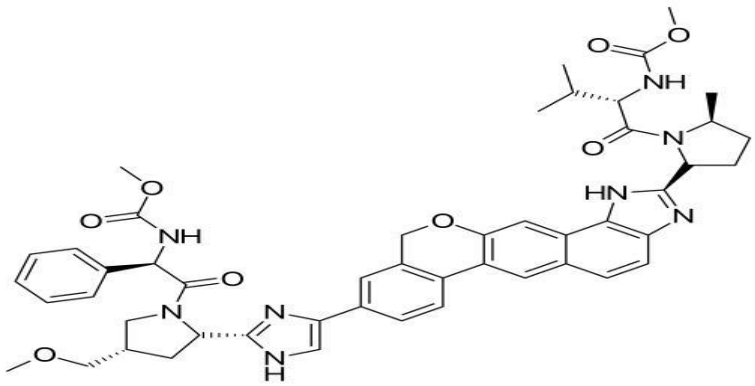


**Figure 2: Chemical Structure of Velpatasvir.**

## 2. MATERIALS AND METHODS

### Apparatus

The chromatography was performed on a Water (Acquity) RP-UPLC instrument equipped with PDA detector and Empower 2 software (Version- EMPOWER SOFTWARE); Purospher Star C18 column (100mm × 2.1 mm id, 2µm particle size, Merck, Germany) was used as stationary phase. Mettler Toledo analytical balance (Germany), pH meter from Lab India, an ultrasonic cleaner, Hot air oven (Lab India), Photo stability chamber were used in the study.

### Reagents andmaterials

Sofosbuvir and Velpatasvir bulk powder was obtained from Spectrum pharma Pvt .Ltd., Hyderabad. All the chemicals used of HPLC grade. The pharmaceutical dosage form (EPCLUSA) was purchased from local pharmacy. The solvents used in this work were of HPLC grade and obtained from Merck Specialties Private Limited, Mumbai. Milli Q Water was used in the bufferpreparation.

### ChromatographicCondition

Separation was achieved by using Purospher Star C18 column (100mm × 2.1 mm id, 2µm particle size, Marck, Germany) as stationary phase with ortho phospharic acid buffer: Acetonitrile (40:60) as a mobile phase and $P^H$ of 2.8 were adjusted by tri ethyl amine at a flow rate of 0.2 ml/min and 6 min run time. Quantification was achieved of Sofosbuvir and Velpatasvir at 250 nm with PDA detector at 25± 2$^o$C temperature condition and 2µl injection volume.

### Preparation of mobilephase

1 ml of ortho phosphoric acid was transferred into 1000 ml volumetric flask. Approximately 100 ml of water was added into the volumetric flask and the volume was made up to 1000 ml with water and the $P^H$was adjusted to 2.8 by using triethylamine and filtered the solution. From this buffer solution 400 ml of solution was withdrawn and mixed with 600 ml of Acetonitrile into separated 1000 ml volumetric flask to make a mobile phase ratio buffer: Acetonitrile (40:60%v/v). Mixture of water and acetonitrile in the ratio of 50: 50 V/V was taken and filtered through 0.45 µ filter under vacuum. This was used as diluents throughout study.

### Preparation of standardsolution

### Preparation of standard stock solution of Sofosbuvir (SOF)

An accurately weighed 40 mg of quantity of SOF reference standard was transferred into 10 ml volumetric flask, dissolved in 3/4$^{th}$ mL mobile phase and sonicated. After this it was diluted up to mark with diluent to get concentration of SOF (400µg/mL).

**Working standard solution of SOF**

400 µg/ml of SOF working standard solution was prepared by diluting 1 ml from above stock solution, made up to 10 ml with diluent into 10 ml volumetric flask**.**

**Preparation of standard stock solution of Velpatasvir (VEL)**

An accurately weighed 10 mg of quantity of VEL reference standardwastransferred into 10 ml volumetric flask, dissolved in 3/4$^{th}$ volume of diluent and sonicated. After this it was diluted up to mark with diluent to get concentration of VEL(100µg/mL).

**Working standard solution of VEL**

100 µg/ml of VEL working standard solution was prepared by diluting 1 ml from above stock solution, made up to 10 ml with diluents into 10 ml volumetric flask**.**

**Calibration curve of SOF and VEL**

**Sofosbuvir**

Aliquots of standard stock solution (400µg/ml) of SOF (0.25, 0.5, 0.75, 0.1, 1.25, 1.5 ml) were transferred into a series of 10 ml volumetric flasks and volume was adjusted to the mark with mobile phase to get concentrations of 100, 200, 300, 400, 500 and 600µg/ml. Solutions were injected into the system with stated chromatographic conditions..The graph of area of peak obtained versus respective concentration was plotted. The mean area and its standard deviation were calculated.

**Velpatasvir**

Aliquots of working standard solution (100µg/ml) of VEL (0.25, 0.5, 0.75, 1.0, 1.25, 1.5 ml) were transferred into a series of 10 ml volumetric flasks and volume was adjusted to the mark with mobile phase to get concentrations of 25, 50, 75 ,100, 125 and 150 µg/ml of VEL. Solutions were injected into the system with stated chromatographic conditions. The graph of area of peak obtained versus respective concentration was plotted. The mean area and its standard deviation were calculated.

**Preparation of Marketed sample solution forAssay**

Assay of SOF and VEL in marketed tablet dosage form (Epclusa) containing label claim of SOF-400 mg and VEL-100mg was carried out. In this assay procedure, 20 tablets were weighed and ground to a fine powder. Weigh the powder equivalent to 40 mg of SOF and 10 mg of VEL was transferred to a 10 ml volumetric flask containing about 3/4$^{th}$ mL mobile phase and sonicated. The solution was diluted up to the mark with mobile phase. The solution was filtered through Whatmann filter paper No. 41. Accurately measured 1.0 ml of solution was transferred to 10 ml volumetric

flask, diluted up to the mark with mobile phase to get final working concentration of SOF (400 µg/ml) and VEL (100 µg/ml). A sample solution was injected under the operating chromatographic condition as described above and responses wererecorded.

## 3. METHOD DEVELOPMENT AND VALIDATION OF HPLC

The proposed analytical method was validated according to ICH guidelines (Q2B) with respect to certain parameters such as system suitability, specificity, linearity, accuracy, precision, robustness and ruggedness.

### 3.1 Specificity

The specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak9. The specificity of the method was determined by observing the interference of any of the possible impurities and excipients.

### 3.2 Linearity

The linearity of the proposed HPLC procedure was evaluated by analysing a series of different concentrations for each of the two analytes and found that the measured peak areas were proportional to concentrations of the analytes. A stock solution of 1000 ppm of two analytes was prepared with diluent. From it, various working standard solutions were prepared in the range of 100 to 600 ppm and 25 to 150 ppm for SOF and VEL respectively and injected into HPLC. It was shown that the selected drugs had linearity in stated range. The calibration plot (peak area versus concentration) was generated by replicate analysis (n=3) at all concentration levels and the linear relationship was evaluated using the least square method. The retention time of standards was 2.124 min for SOF and 3.334 min for VEL. A typical HPLC chromatogram of the standard mixtures is shown in Fig.5

### 3.3 Accuracy

Accuracy study was performed for 50%, 100% and 150 % for SOF and VEL in terms of % recovery. Standard and sample solutions were injected in to HPLC system in triplicate and percentage recoveries of SOF and VEL were calculated. The area of each level was used for calculation of % recovery.

### 3.4 Precision

The precision of the method was ascertained from the peak area obtained by actual determination of six replicates of 400ppm and 100ppm of Sofosbuvir, Velpatasvir respectively. The precision of the assay was also determined in terms of intra- and inter-day variation in the peak areas of a set of drug solutions on three different days. The intra and inter-day variation in the peak area of the drug solution was calculated in terms of relative standard deviation (RSD). The system precision values and method precision values are shown in table no 3&4.

### 3.5 Limit Of Detection And Quantification

The limit of detection values for SOF and VEL were 0.15 ppm, and 0.28 ppm, respectively. The limit of quantification values for SOF and VEL were 0.46 ppm, and 0.84 ppm, respectively. The above two parameters are within the range as per the recommendations of USP,2011.

### 3.6 Robustness

Robustness of the developed method was studied by evaluating the influence of small deliberate variations in procedure variables like flow rate (±5%) and change in wave length (±5nm). The robustness was performed for the flow rate variations from 0.8ml/min to 1.2ml/min and the method is robust only in less flow condition and even by change in the mobile phase ±5%. The results are shown in Table 5.

### 3.7 System Suitability

To ascertain certain system suitability and its effective, the test parameters were checked by repetitively injecting the freshly prepared standard stock solutions at the concentration level 400ppm and 100ppm of Sofosbuvir, Velpatasvir respectively to check the reproducibility of the system.

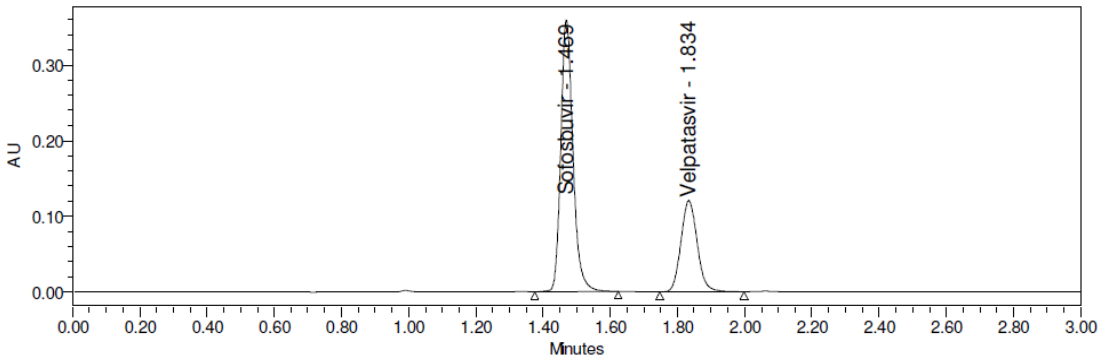
## 4. Results and Discussions

The present investigation reported is a new RP-HPLC method development and validation of simultaneous estimation of SOF and VEL. In order to get the optimized RP-HPLC method, various mobile phases and columns were used. From several trials final method is optimized with the following conditions.

### 4.1 METHOD DEVELOPMENT

The mobile phase consists of 0.1% ortho-phosphoric acid buffer and acetonitrile in the ratio of 40:60 %v/v and the column used was Purospher Star C18 column (100mm × 2.1 mm id, 2µm particle size, Marck, Germany) as stationary phase The flow rate was adjusted to 0.2ml/min. The instrument was operated at an ambient temperature. The UV detection was achieved at 250nm.

**Fig.1: Standard Chromatogram of Sofosbuvir and Velpatasvir**



**4.2 METHOD VALIDATION**

Since there is no interference of the other substances in the retention time of the analytical peak. Hence this method was said to be a specific. The linearity was determined as linearity regression of the claimed analyte concentration of the range 100 to 600 ppm, and 25 ppm to 150 ppm for SOF and VEL respectively. The correlation coefficient was found to be 0.999, and 0.999 for SOF and VEL respectively. Hence the results were obtained within the limit.

**Table No. 1: Linearity results of SOF and VEL**

| % Level of concentration | Sofosbuvir | | Velpatasvir | |
|---|---|---|---|---|
| | Conc. (µg/ml) | Peak Area | Conc. (µg/ml) | Peak Area |
| 25 | 100 | 223249 | 25 | 98398 |
| 50 | 200 | 441698 | 50 | 203585 |
| 75 | 300 | 672602 | 75 | 306369 |
| 100 | 400 | 915361 | 100 | 408733 |
| 125 | 500 | 1111527 | 125 | 498495 |
| 150 | 600 | 1337858 | 150 | 604828 |

**Table No. 2: Regression characteristics of SOF and VEL**

| % Level of concentration | Sofosbuvir | | Velpatasvir | |
|---|---|---|---|---|
| | Conc. (µg/ml) | Peak Area | Conc. (µg/ml) | Peak Area |
| 25 | 100 | 223249 | 25 | 98398 |
| 50 | 200 | 441698 | 50 | 203585 |
| 75 | 300 | 672602 | 75 | 306369 |
| 100 | 400 | 915361 | 100 | 408733 |
| 125 | 500 | 1111527 | 125 | 498495 |
| 150 | 600 | 1337858 | 150 | 604828 |

**Fig. 2: Linearity graph of Sofosbuvir**

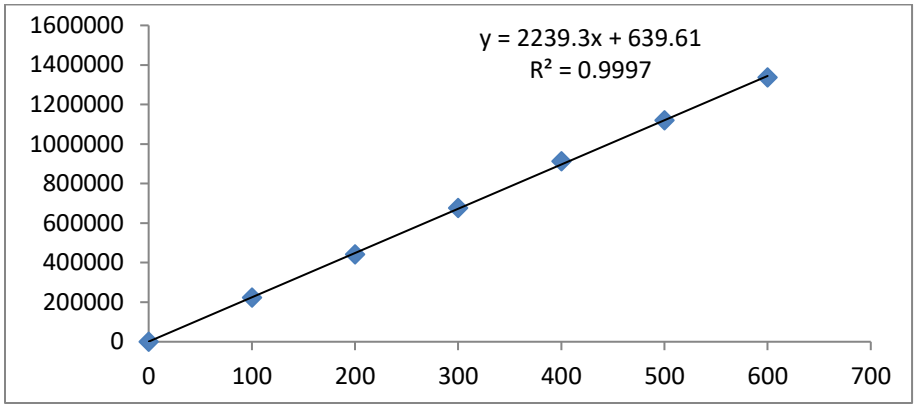
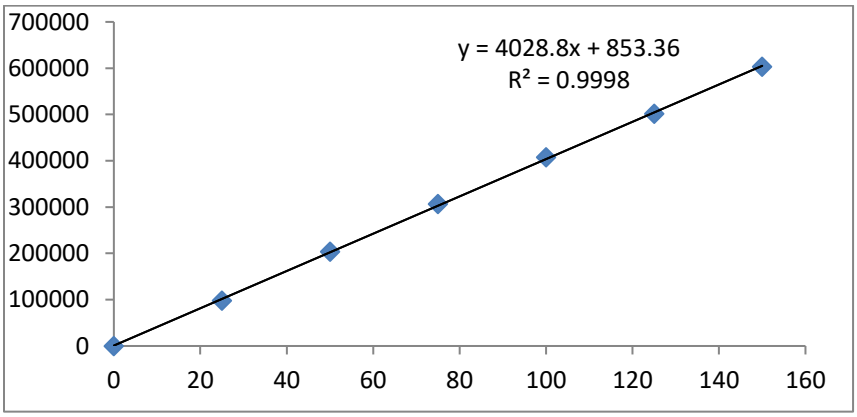



**Fig. 3: Linearity graph of Velpatasvir**




**ACCURACY OF THE PROPOSED HPLC METHOD:**

Accuracy of the developed method was determined by standard addition method (n=average of 3 analytes). In this method, known amounts of Sofosbuvir and Velpatasvir were supplemented to the previously analysed

sample solution and then experimental and true values were compared. Three levels were made corresponding to 50%, 100% and 150% of the nominal analytical concentration. The % recovery was found to be 98.4% for sofosbuvir and 99.5% for Velpatasvir and the results were tabulated in Table no.3

**Table No.3: Accuracy results of SOF and VEL in combined tablet form**

| Drug | Amount taken | Amount added | | Amount recovered | % Amount found | % Mean |
|---|---|---|---|---|---|---|
| | | % | PPM | | | |
| SOF | 400 | 50 | 200 | 197.13 | 99.26 | 1.45 |
| | 400 | 100 | 400 | 398.47 | 98.86 | 0.77 |
| | 400 | 150 | 600 | 599.25 | 99.77 | 1.04 |
| VEL | 100 | 50 | 50 | 49.59 | 99.37 | 1.39 |
| | 100 | 100 | 100 | 99.56 | 99.75 | 0.41 |
| | 100 | 150 | 150 | 150.04 | 100.26 | 0.27 |

The precision of the method was ascertained from determinations of peak areas of six replicates of sample solution. The %RSD for method precision was found to be 0.4 and 0.4 for SOF and VEL respectively and the results were tabulated in Table no.4 & 5.

**Table No. 4: Method Precision values for SOF and VEL tablet sample solutions**

| No. of Injection | Retention time (mins) | | Peak Area | |
|---|---|---|---|---|
| | Sofosbuvir | Velpatasvir | Sofosbuvir | Velpatasvir |
| 1 | 1.468 | 1.468 | 911448 | 406724 |
| 2 | 1.469 | 1.469 | 904113 | 404298 |
| 3 | 1.469 | 1.469 | 913002 | 414979 |
| 4 | 1.470 | 1.470 | 901158 | 403276 |
| 5 | 1.472 | 1.472 | 910097 | 409155 |
| 6 | 1.472 | 1.472 | 901097 | 409035 |
| Statistical Parameters | Mean | | 918791 | 906819 |
| | SD | | 6407.6 | 5338.8 |
| | %RSD | | 0.7 | 0.6 |

**Table No. 5: System Precision values for SOF and VEL tablet sample solutions**

| No. of Injection | Retention time (mins) | | Peak Area | |
|---|---|---|---|---|
| | Sofosbuvir | Velpatasvir | Sofosbuvir | Velpatasvir |
| 1 | 1.468 | 1.468 | 919401 | 414829 |
| 2 | 1.469 | 1.469 | 927403 | 411424 |
| 3 | 1.469 | 1.469 | 913075 | 405428 |
| 4 | 1.470 | 1.470 | 912893 | 408882 |

| | | | | |
|---|---|---|---|---|
| 5 | 1.472 | 1.472 | 914491 | 414325 |
| 6 | 1.472 | 1.472 | 925484 | 407774 |
| **Statistical Parameters** | **Mean** | | 918791 | 410444 |
| | **SD** | | 6407.6 | 3741.1 |
| | **%RSD** | | 0.7 | 0.9 |

The limit of detection values for SOF and VEL were 0.15 ppm, and 0.28 ppm, respectively. The limit of quantification values for SOF and VEL were 0.46 ppm, and 0.84 ppm, respectively. The values were shown in Table no.6

**Table No.6 LOD and LOQ values of SOF and VEL**

| **Drug name** | **LOD** | **LOQ** |
|---|---|---|
| **Sofosbuvir** | 0.21 | 0.64 |
| **Velpatasvir** | 0.06 | 0.18 |

The robustness was carried out with minor but deliberate changes in parameters i.e., mobile phase, column temperature, and flow rate as presented in Table 7.

**Table 7: Robustness study of Sofosbuvir.**

| **Parameter** | **Optimized condition** | **Used condition** | **Peak area** | **Retention Time** | **Plate Count** | **Tailing factor** |
|---|---|---|---|---|---|---|
| Flow rate (±0.1ml/min) | 1ml/min | 0.9ml/min | 923257 | 1.576 | 7524 | 1.16 |
| | | 1ml/min | 904118 | 1.577 | 7563 | 1.15 |
| | | 1.1ml/min | 903924 | 1.577 | 7579 | 1.15 |
| Column temp. ($\pm5^0$c) | $30^0$c | $25^0$c | 928258 | 1.577 | 7489 | 1.15 |
| | | $30^0$c | 905215 | 1.579 | 7511 | 1.16 |
| | | $35^0$c | 916099 | 1.579 | 7521 | 1.15 |
| Mobile phase Composition (5%v/v) | 40:60 | 35:65 | 913555 | 1.367 | 7407 | 1.18 |
| | | 40:60 | 895940 | 1.367 | 7438 | 1.18 |
| | | 45:55 | 907909 | 1.367 | 7447 | 1.18 |

**Table 8: Robustness study of Velpatasvir.**

| Parameter | Optimized condition | Used condition | Peak area | Retention Time | Plate Count | Tailing factor |
|---|---|---|---|---|---|---|
| Flow rate (±0.1ml/min) | 1ml/min | 0.9ml/min | 409079 | 1.660 | 6490 | 1.04 |
| | | 1ml/min | 400627 | 1.661 | 6512 | 1.04 |
| | | 1.1ml/min | 400375 | 1.661 | 6494 | 1.03 |
| Column temp. ($\pm5^0$c) | $30^0$c | $25^0$c | 410695 | 1.661 | 6548 | 1.03 |
| | | $30^0$c | 400196 | 1.662 | 6548 | 1.03 |
| | | $35^0$c | 406653 | 1.662 | 6539 | 1.04 |
| Mobile phase Composition (5%v/v) | 40:60 | 35:65 | 400988 | 1.660 | 6893 | 1.06 |
| | | 40:60 | 395728 | 1.661 | 6883 | 1.06 |
| | | 45:55 | 403241 | 1.661 | 6867 | 1.06 |

The system suitability parameters like theoretical plates (N), tailing factor (T) were calculated and were found to be more than 2000 and not more than 2 and ascertained that proposed RP-HPLC method was accurate and precise.

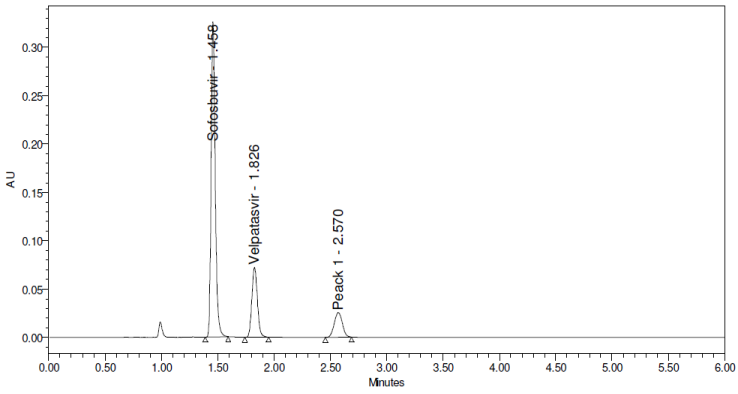
**Table 9: System suitability test parameters**

| Parameter | Sofosbuvir | Velpatasvir |
|---|---|---|
| **Retention time** | 1.472 | 1.836 |
| **Theoretical plates** | 8403 | 6852 |
| **Tailing factor** | 1.24 | 1.11 |

**Forced Degradation Studies:**

The chromatograms of the stressed samples were evaluated for peak purity as shown in the fig.4. These stress degradation results of Sofosbuvir and Velpatasavir are reported in Tables 10.

**Fig.4. UPLC Chromatogram of Acid Degraded sample**




| S.No. | Forced degradation condition | % degradation of Sofosbuvir | % degradation of Velpatasvir | Peak purity |
|---|---|---|---|---|
| **1** | Acid stress | 4.62 | 4.64 | Passes |
| **2** | Alkali stress | 2.70 | 2.71 | Passes |
| **3** | Oxidation stress | 1.51 | 1.78 | Passes |
| **4** | Thermal stress | 0.90 | 0.90 | Passes |
| **5** | Photolytic Stress | 0.74 | 0.84 | Passes |
| **6** | Neutral stress | 0.94 | 0.79 | Passes |

From the above study, a degradent was observed at retention time of 2.570 mins in Acid, 2.572 mins in Alkali, and 0.782 mins in Peroxide degradation studies. No significant degradent was observed under Thermal, Photolytic and Neutral degradarion studies were degraded below 5% without any major degradents.

## 5. CONCLUSION

The results of this investigation reveals that by applying the proposed UPLC method, the retention times of Sofosbuvir and Velpatasavir were found to be 1.457 and 1.824mins respectively. Quantitative linearity was obeyed in the concentration range of 100-600 µg/ml and 25 – 150 µg/ml with correlation coefficient value of 0.999. The %RSD values obtained from the precision studies were also found to be less than 2, which indicates precise method. The high % recoveries indicate that the proposed method was highly accurate. The low values of LOD and LOQ indicates the high sensitivity of the proposed method. The absence of

interfering peaks observed in the chromatogram of blank and placebo interference studies indicates specific method and degradants formed during stress degradation studies were also well separated and not interfere with estimation of the drugs by the proposed stability indicating UPLC method. From this study it is concluded that the proposed stability indicating UPLC method was found to be simple, accurate, precise, rapid and useful for the routine analysis of Sofosbuvir and Velpatasavir in bulk and pharmaceutical dosage forms. The obtained results were satisfactory as per ICH guidelines.

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