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DEVELOPMENT AND VALIDATION FOR ESTIMATION OF SOFOSBUVIR IN BULK AND PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC METHOD

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Abstract:

A rapid and precise Reverse Phase High Performance Liquid Chromatographic method has been developed for the validated of Sofosbuvir, in its pure form as well as in tablet dosage form. Chromatography was carried out on a Hypersil C18(4.6×150mm, 5µ) column using a mixture of phosphate Buffer and Acetonitrile (45:55 v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 260nm. The retention time of the Sofosbuvir was 3.166±0.02min respectively. The method produce linear responses in the concentration range of 35-420µg/ml of Sofosbuvir. The method precision for the determination of assay was below2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: *Sofosbuvir, RP-HPLC, validation.*

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INTRODUCTION:

Chromatography:

The chromatography was discovered by Russian Chemist and botanist Micheal Tswett (1872-1919) who first used the term chromatography (colour writing derived from Greek for colour – Chroma , and write – graphein) to describe his work on the separation of coloured plant pigments into bands on a column of chalk and other material such as polysaccharides, sucrose and insulin.Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system.The adsorbent material, or stationary phase, first described by Russian scientist named Tswett in 1906, has taken many forms over the years, including paper, thin layers of solids attached to glass plates, immobilized liquids, gels, and solid particles packed in columns. The flowing component of the system, or mobile phase, is either a liquid or a gas. Concurrent with development of the different adsorbent materials has been the development of methods more specific to particular classes of analytes. In general, however, the trend in development of chromatography has been toward faster, more efficient.In his early papers of Tswett (1906) stated that chromatography is a method in which the component of a mixture are separated on an adsorbent column in a flowing system. Chromatography has progressed considerably from Tswett's time and now includes a number of variations on the basic separation process.Chromatography is a physical method of separation in which the component to be separated are distributed between two phases of which in stationary while other moves in a definite direction (IUPAC).

1.1.2. Chromatographic Process:

Chromatographic separations are based on a forced transport of the liquid (mobile phase) carrying the analyst mixture through the porous media and the differences in the interactions at analyst with the surface of this porous media resulting in different migration times for a mixture components. In the above definition the presence of two different phases is stated and consequently there is an interface between them. One of these phases provides the analyst transport and is usually referred to as the mobile phase, and the other phase is immobile and is typically referred to as the stationary phase. A mixture of components, usually called analyst, are dispersed in the mobile phase at the molecular level allowing for their uniform transport and interactions with the mobile and stationary phases. High surface area of the interface between mobile and stationary phases is essential for space discrimination of different components in the mixture. analyst molecules undergo multiple phase transitions between mobile phase and adsorbent surface. Average residence time of the molecule on the stationary phase surface is dependent on the interaction energy. For different molecules with very small interaction energy difference the presence of significant surface is critical since the higher the number of phase transitions that analyst molecules undergo while moving through the chromatographic column, the higher the difference in their retention. The nature of the stationary and the mobile phases, together with the mode of the transport through the column, is the basis for the classification of chromatographic methods. [3,4]

1.1.3. Types of Chromatography:

The mobile phase could be either a liquid or a gas, and accordingly we can subdivide chromatography into Liquid Chromatography (LC) or Gas Chromatography (GC). Apart from these methods, there are two other modes that use a liquid mobile phase, but the nature of its transport through the porous stationary phase is in the form of either (a) capillary forces, as in planar chromatography (also called Thin-Layer Chromatography, TLC), or (b) electro osmotic flow, as in the case of Capillary Electro Chromatography(CEC). [5,6]

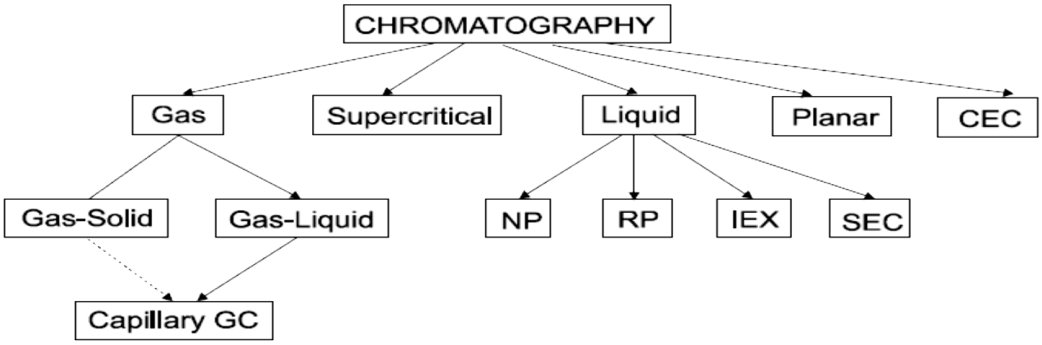

Figure No: 1.1 Showing flow chart for classification of chromatography

Principles and classification of chromatography

SELECTION CRITERIA FOR SOFOSBUVIR:

Technique	Stationary Phase	Mobile Phase	Format	Principal sorption mechanism
Paper chromatography (PC)	Paper (cellulose)	Liquid	Planar	Partition (adsorption, ion-exchange, exclusion)
Thin-layer chromatography (TLC)	Silica, cellulose, ion-exchange, resin,controlled porosity solid	Liquid	Planar	Adsorption (partition,ion-exchange, exclusion)
Gas-liquid chromatography (GLC)	Liquid	Gas	Column	Partition
Gas-Solid chromatography (GSC)	Solid	Gas	Column	Adsorption
Classical Liquid-Liquid Chromatography (LLC)	Liquid	Liquid	Column	Partition
HighPerformance Liquid Chromatography (HPLC)	Liquid	Liquid	Column	Modified Partition
High Performance Thin-LayerChromatography(HPTLC)	Liquid	Liquid	Planar	Modified Partition
Ion Exchange Chromatography(IEC)	Solid	Liquid	Column	Ion Exchange

Sofosbuvir is used primarily to treat hepatitis C and viral hemorrhagic fevers. It is possible to select a sofosbuvirresistant mutant of HCV that can replicate to levels similar to wild type virus grown without sofosbuvir. Analysis of the mutations responsible for the sofosbuvir resistance may aid in understanding the mechanism of action of sofosbuvir.

Sofosbuvirhas proven to be effective for the treatment of HCV chronically infected patients when combined with interferon. Given the side effects associated with intravenous injections of interferon, an interferon-free regimen for the treatment of HCV infections is highly desirable. The study of HCV resistance tosofosbuvir could be specially important for an interferon-free era. In this report, we show that it is possible to select a sofosbuvirresistant mutant of HCV *in vitro* that can replicate to similar levels to virus grown without sofosbuvir. Analysis of the mutations responsible for thesofosbuvir resistance phenotype, currently underway, may aid in understanding the mechanism of action of sofosbuvir.

Sofosbuvir is a synthetic guanosine analogue that has shown antiviral activity against a broad range of viruses. The aerosol formulation of the drug is approved for use against respiratory syncytial virus. The precise antiviral mechanism of action of the drug is unknown but may involve: decrease of intracellular quanosine triphosphate that suppresses synthesis of viral nucleic acid; formation of defective or absent 5' cap structure, and in efficient translation of viral RNA; and suppression of viral polymerase.

INTRODUCTION TO DISEASE:

Hepatitis C virus (HCV) infection is associated with renal manifestations, such as membranoproliferative glomerulonephritis (MPGN) with or without cryoglobulinaemia, membranous glomerulonephritis (MGN) and focal segmental glomerulosclerosis (FSGS). Standard treatment for HCV is interferon and sofosbuvir, but in renal insufficiency sofosbuvirhas been contraindicated due to fear of side effects.

Hepatitis C virus (HCV) is a major cause of acute and chronic hepatitis throughout the world. Several extrahepatic manifestations, including glomerulonephritis, have been reported to be associated with this type of infection. Cryoglobulinaemic and non-cryoglobulinaemic membranoproliferative glomerulonephritis (MPGN) and membranous nephropathy (MN) are the commonest lesions associated with HCV. Results of treatment of these patients with interferon therapy have been disappointing, since relapse of the

viraemia and subsequent relapse of the renal disease are major problems. Combination of interferon with sofosbuvir in patients with chronic liver disease has been shown to increase the rate of sustained response.

DRUG PROFILE:

Sofosbuvir is guanosine (ribonucleic) analog used to stop viral RNA synthesis and viral mRNA capping, thus, it is a nucleoside inhibitor. Its brand names include Copegus, Rebetol, Ribasphere, Vilona, and Virazole. It is an anti-viral drug used for severe RSV infection [1]; hepatitis C infection, including if persistent,[2] and often in combination with peginterferon alfa-2b orpeginterferon alfa-2a; as well as some other viral infections. Sofosbuvir is aprodrug, which when metabolized resembles purine RNA nucleotides. In this form it interferes with RNA metabolism required for viral replication. How it exactly affects viral replication is unknown; many mechanisms have been proposed for this but none of these has been proven to date. Multiple mechanisms may be responsible for its actions.

AIM AND OBJECTIVE:

- Review of literature for Sofosbuvir gave information regarding its physical and chemical properties, various analytical methods that were conducted alone and in combination with other drugs.
- In view of the need for a suitable RP-HPLC method for routine analysis of Sofosbuvir in formulations, attempts were made to develop simple, precise and accurate analytical method for estimation of Sofosbuvir and extend it for their determination in formulation.
- Validation is a necessary and important step in both framing and documenting the capabilities of the developed method.
- The utility of the developed method to determine the content of drug in commercial formulation was also demonstrated. Validation of the method was done in accordance with USP and ICH guideline for the assay of active ingredient. The method was validated for parameters like system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection and limit of quantification. This method provides means to quantify the component. This proposed method was suitable for the analysis of Pharmaceutical dosage forms.

Primary Objective Of Proposed Work Is: To develop new simple, sensitive, accurate and economical analytical method for the estimation of Sofosbuvir.

To validate the proposed method in accordance with USP and ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the Sofosbuvir in dosage form.

RESULTS AND DISCUSSION:

Development of RP-HPLC Method For Sofosbuvir:

Trails

Trail 1:

Column : ODS C18 (4.6 × 250mm) 5µ
Column temperature :Ambient
Wavelength :260nm
Mobile phase ratio :Water (100%) V/V
Flow rate :0.5ml/min
Injection volume : 20µl
Run time : 7minute

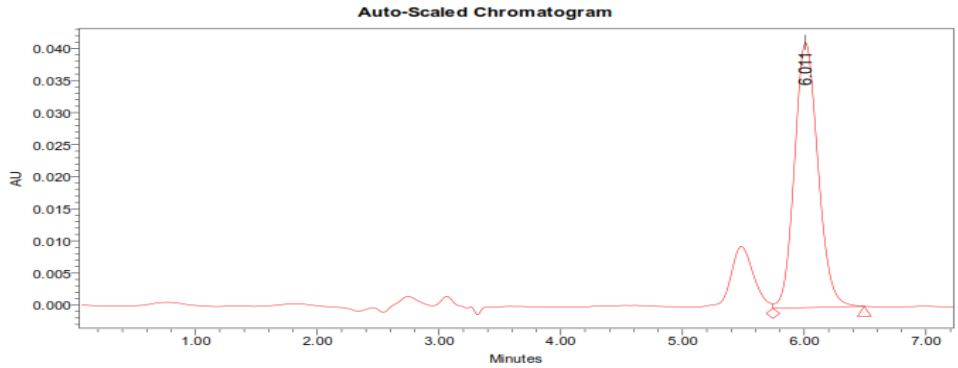

Figure 1: Chromatogram for trail 1

Table 1: Peak results for trail 1

s.no	Peak name	Rt (min)	Area	Height	Usp tailing	Usp plate cont
1	Sofosbuvir	3.166	55392	41393	1.6	849

Trail 2:
Column : ODSC18 (4.6 × 250mm) 5µ
Column temperature : Ambient
Wavelength : 260nm
Mobile phase ratio : Phosphate buffer:ACN (45:55) V/V
Flow rate : 0.8ml/min
Injection volume : 20µl
Run time : 8minutes

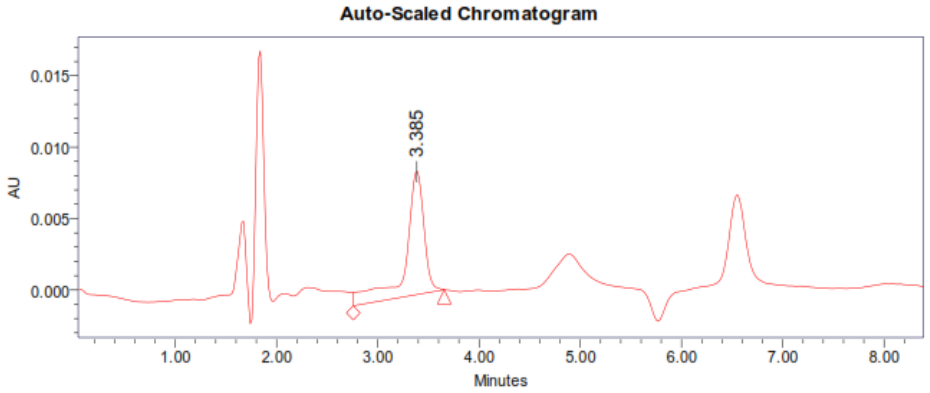

Figure 2: Chromatogram for trail 2

Table 2: Peak results for trail 2

s.no	Peak name	Rt (min)	Area	Height	Usp tailing	Usp plate count
1	sofposbuvir	3.166	110280	8642	Not detectable	2800

Trail 3:
Column : XBridge C18 (4.6 × 150mm) 5µ
Column temperature :Ambient
Wavelength : 260nm
Mobile phase ratio : Phosphate buffer:ACN (45:55) V/V
Flow rate : 0.9ml/min
Injection volume : 20µl
Run time : 6minutes

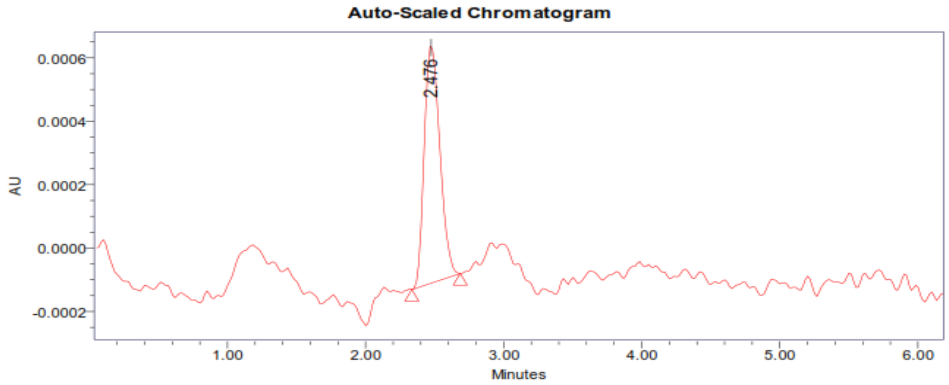

Figure 3: Chromatogram for trail 3

Table 3: Peak results for trail 3

s.no	Peak name	Rt (min)	Area	Height	Usp tailing	Usp plate count
1	Sofosbuvir	3.166	5909	753	1.36	2213

CHROMATOGRAM FOR BLANK:

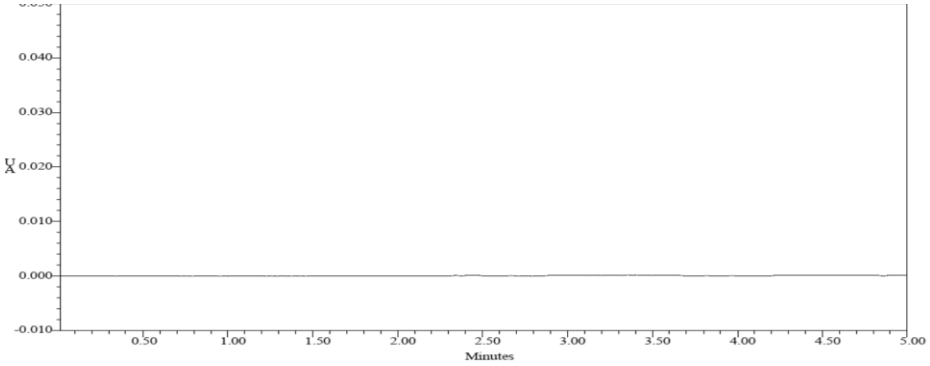

Figure 4: Chromatogram Showing Blank (Mobile Phase Preparation)

Chromatogram for Standard:

Mobile phase ratio :Phosphate buffer : Acetonitrile (45:55 v/v)
Column : Apollo C18 (4.6×150mm) 5µ
Column temperature : Ambient
Wavelength :260nm
Flow rate : 1.0ml/min
Injection volume : 10µl
Run time : 5minutes

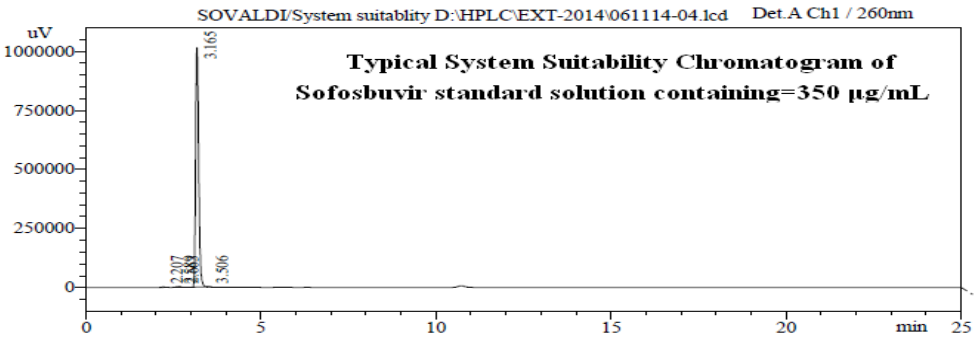

Figure 5: Chromatogram for Standard

Table 4: Peak results for Optimized Chromatogram (Standard)

S.no	Name	Rt (min)	Area	Height	Usp tailing	Usp plate count
1	Sofosbuvir	3.166	625172	95254	1.36	9544

Chromatogram for Sample:

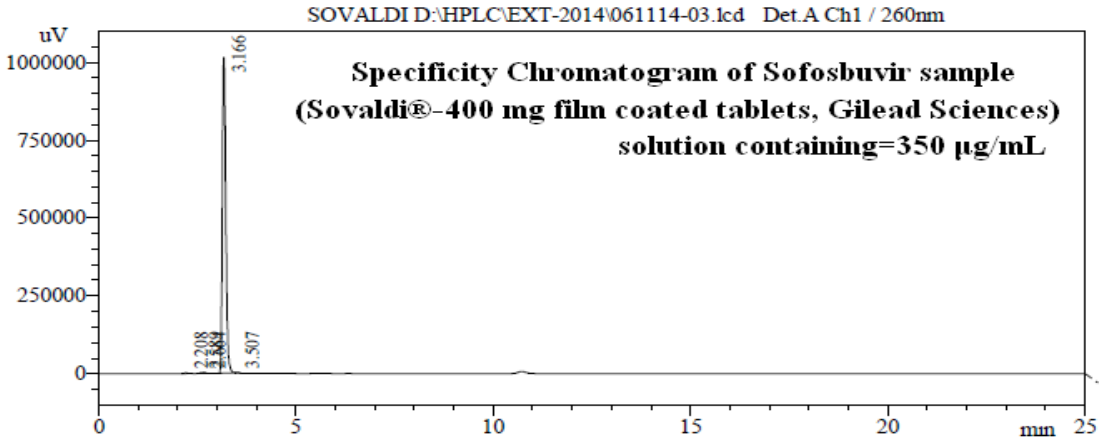

Figure 6: Chromatogram for Sample

Table 5: Peak results for Optimized Chromatogram (Sample)

s.no	Name	Rt (min)	Area	Height	Usp tailing	Usp plate count
1	Sofosbuvir	3.166	6232590	95210	1.36	9573

Method Validation

4.1. SUITABILITY: The system suitability tests were carried out on freshly prepared standard stock solution of Sofosbuvir. The system was suitable for use, the tailing factors for Sofosbuvir were 1.36and
USP theoretical plates were found to be significantly high around 5414.269.

Table 6: Results of System Suitability for Sofosbuvir

s.no	Name	Rt (min)	Area	Height	Usp plate count	Usp tailing
1	Sofosbuvir	3.166	6241725	92251	9217	1.36
2	Sofosbuvir	3.168	6242365	92274	9644	1.36
3	Sofosbuvir	3.164	6233791	92291	9816	1.36
4	Sofosbuvir	3.167	6236755	92183	9017	1.36
5	Sofosbuvir	3.165	6232983	92291	9374	1.36
mean			6237240			
Std dev			4089			
%rsd			0.066			

4.2.Linearity:

Aliquots of standard Sofosbuvir stock solution were taken in different 10 ml volumetric flasks and diluted up to the mark with the mobile phase such that the final concentrations of Sofosbuvir are in the range of 35-420µg/ml. Each of these drug solutions (20 µL) was injected three times into the column, and the peak areas and retention times were recorded. Evaluation was performed with PDA detector at 260 nm and a Calibration graph was obtained by plotting peak area versus concentration of Sofosbuvir. The linearity Chromatograms presented in fig.

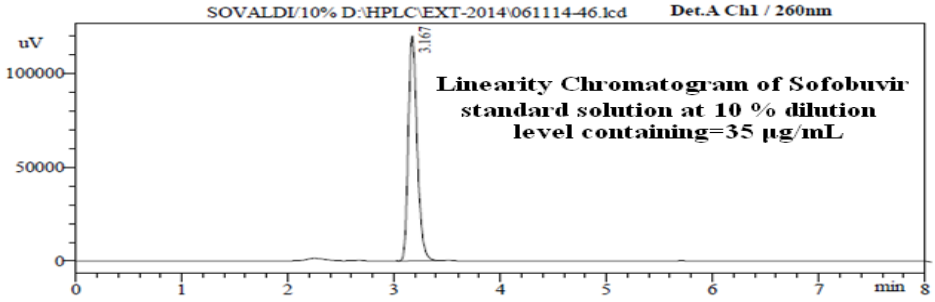

Figure 7: Chromatogram showing Linearity level-1

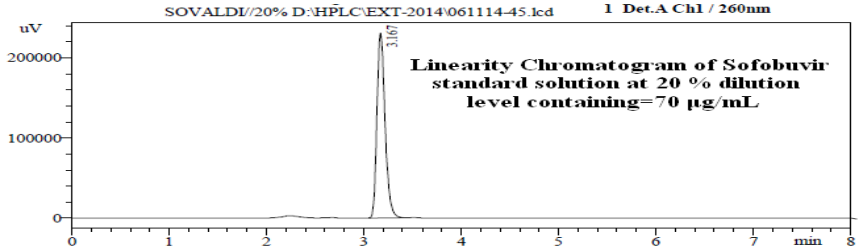

Figure 8: Chromatogram showing Linearity level-2

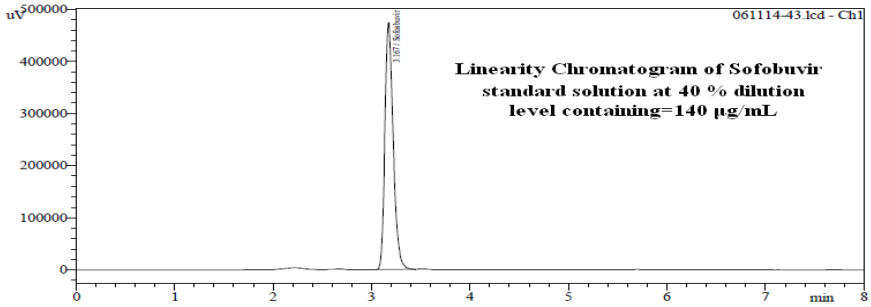

Figure 9: Chromatogram showing linearity level-3

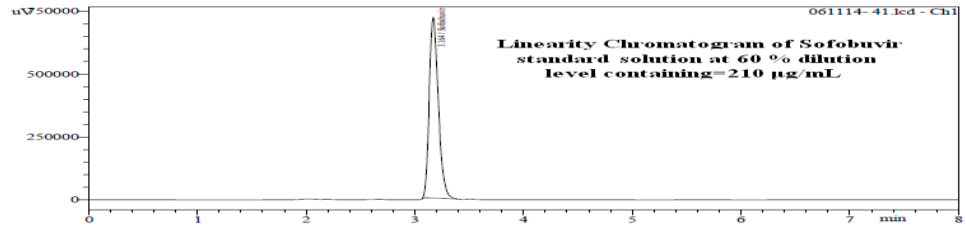

Figure 10: Chromatogram showing linearity level-4

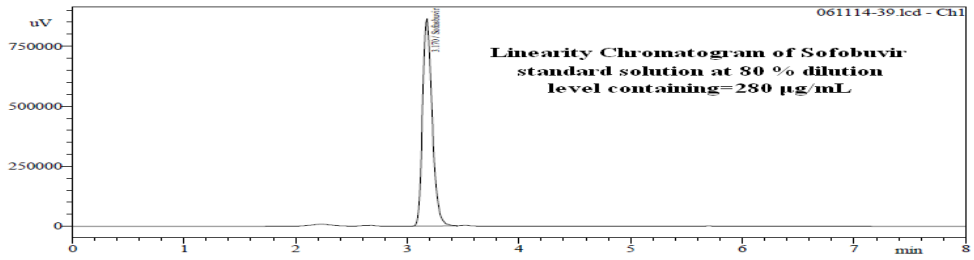

Figure 11: Chromatogram showing linearity level-5

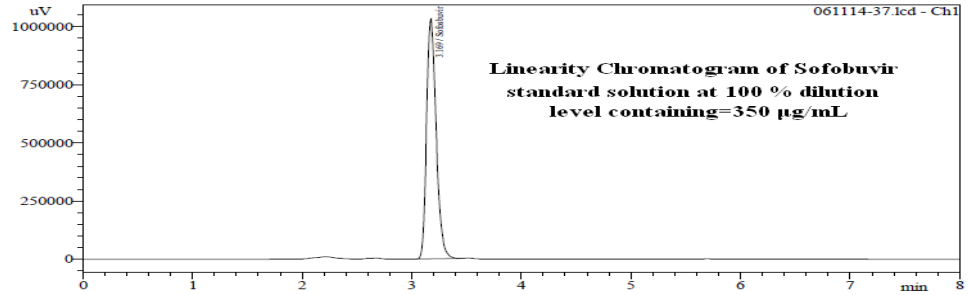

Figure 12: Chromatogram showing linearity level-6

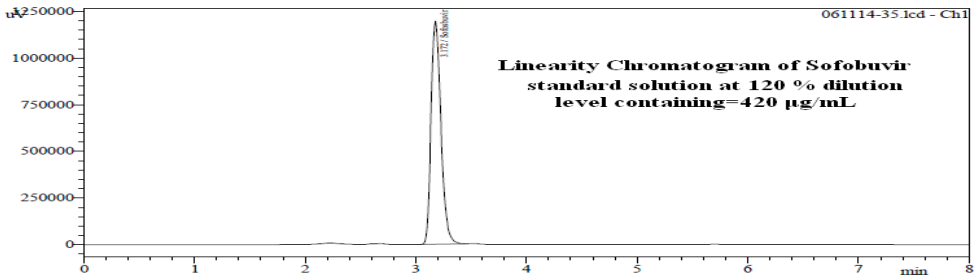

Figure 13: Chromatogram showing linearity level-7

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY:

Table 7: Calibration of Sofosbuvir

Concentration of drug (µg/mL)	Retention time(min)	Peak Area
35	3.167	698762
70	3.168	1534217
140	3.169	2791236
210	3.166	4089902
280	3.170	5180679
350	3.170	6315827
420	3.172	7486081

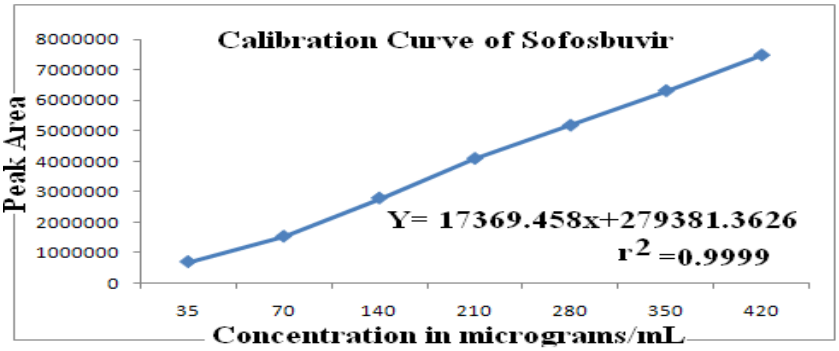

Figure 14: Linearity Plot

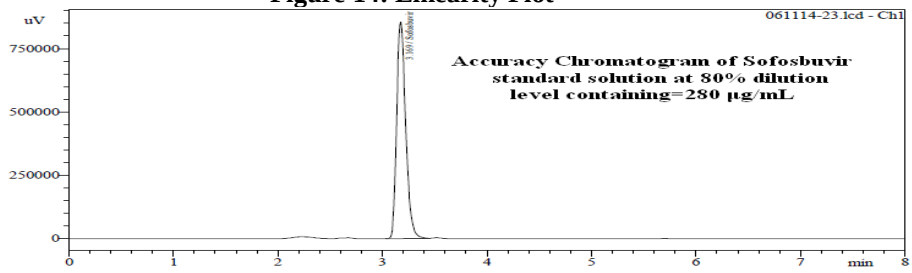

Figure 15: Chromatogram for Accuracy 80% Standard injection

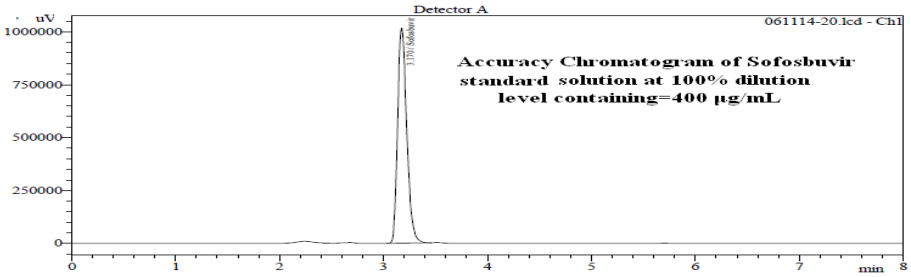

Figure 16: Chromatogram for Accuracy 100% Standard injection

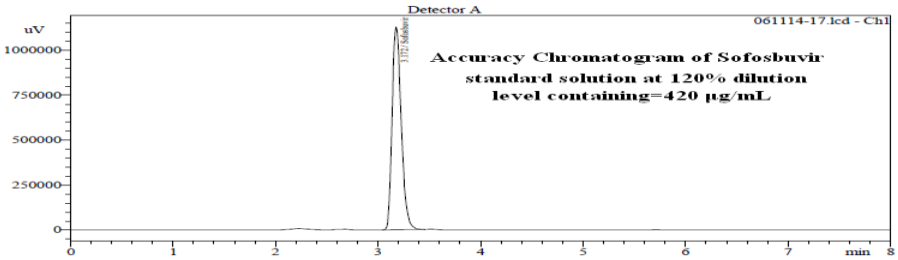

Figure 17: Chromatogram for Accuracy 120% Standard injection

Table 8: Results of Accuracy for Standard injections

S.no	Name	%Concentration	Rt (min)	Area	Height	Usp Tailing	Usp plate count
1	Sofosbuvir	80%	3.166	5127921	71944	1.36	9772
2	Sofosbuvir	100%	3.167	6224163	92210	1.36	9184
3	Sofosbuvir	120%	3.168	7012659	170368	1.36	9754

Accuracy80% (Sample):

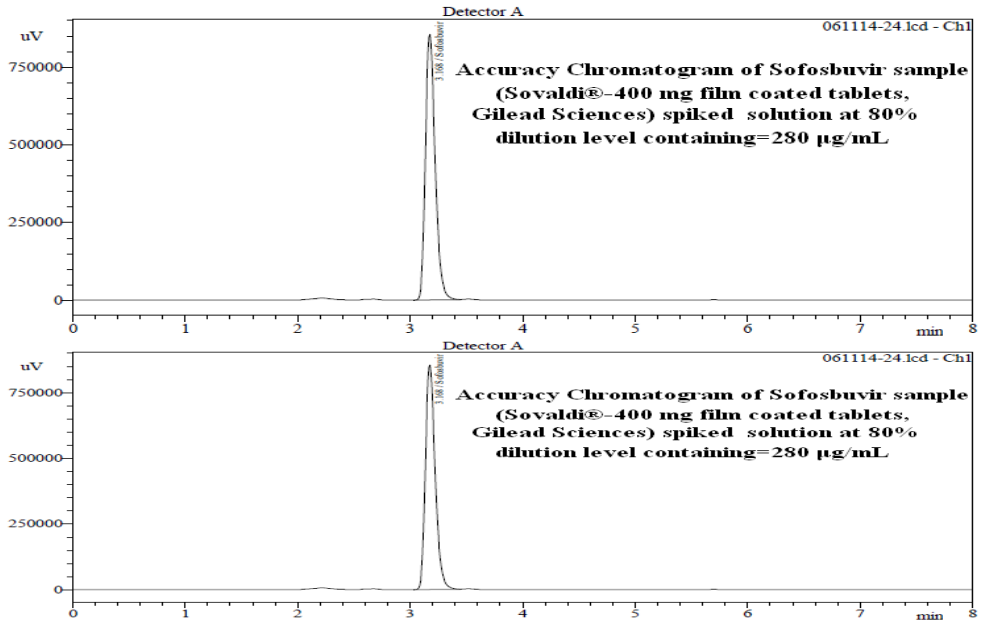

Figure 18: Chromatogram showing Accuracy-80% injection-1

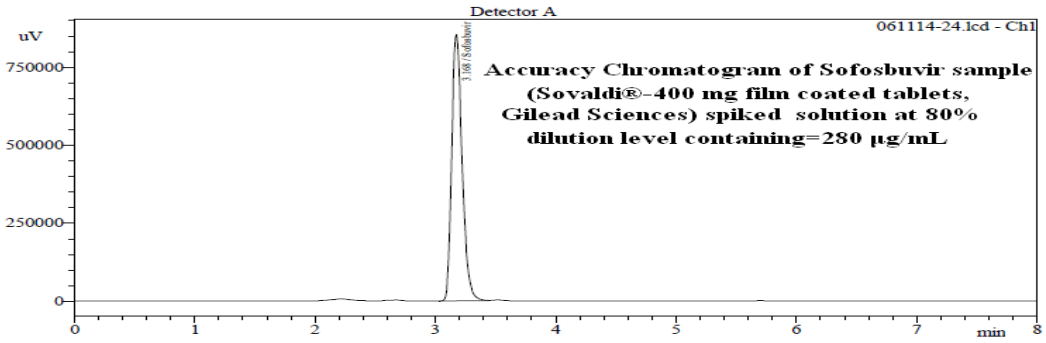

Figure 19: Chromatogram showing Accuracy-80% injection-2

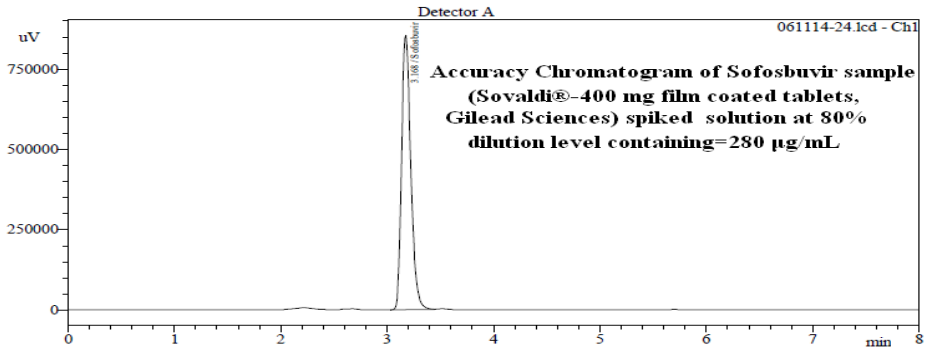

Figure 20: Chromatogram showing Accuracy-80% injection-3

Table 9: Results of Accuracy for Sample injections (Conc.80%)

S.no	Name	Rt (min)	Area	Height	Usp tailing	Usp plate count
1	Sofosbuvir	3.166	5677448	69943	1.36	9585
2	Sofosbuvir	3.168	5680902	71944	1.36	9772
3	Sofosbuvir	3.168	5686669	70928	1.36	9374

Accuracy100% (Sample):

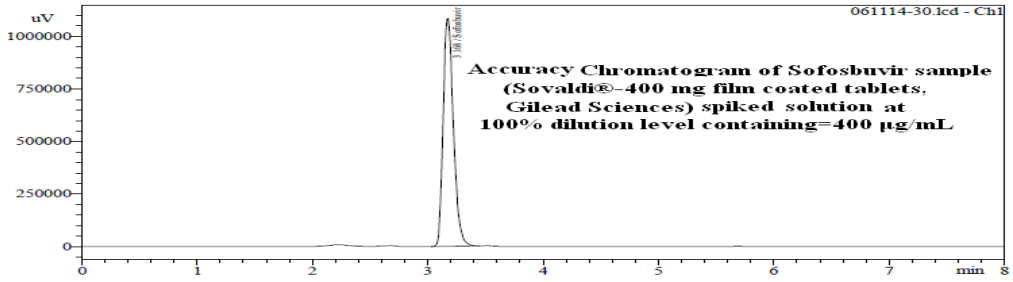

Figure 21: Chromatogram showingAccuracy-100% injection-1

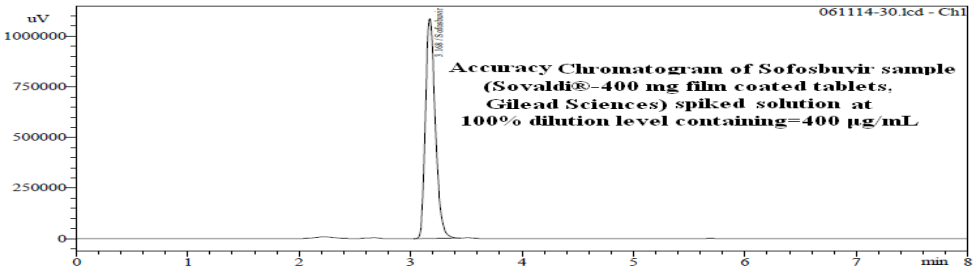

Figure 22: Chromatogram showing Accuracy-100% injection-2

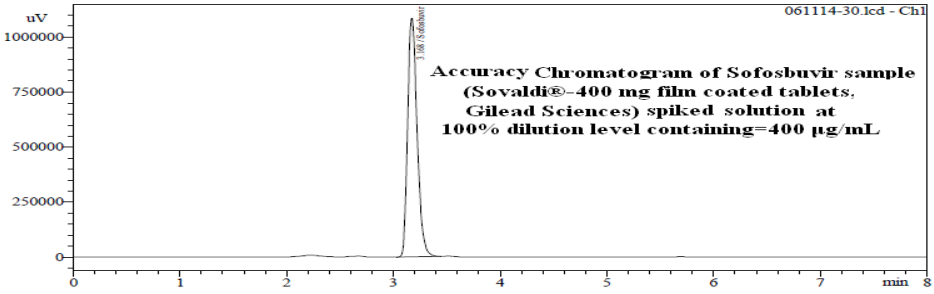

Figure 23: Chromatogram showing Accuracy-100% injection-3

Table 10: Results of Accuracy for Sample injections (Conc.100%)

S.no	Name	Rt (min)	Area	Height	Usp tailing	Usp plate count
1	Sofosbuvir	3.167	6717889	92274	1.36	9744
2	Sofosbuvir	3.166	6709953	92265	1.36	9855
3	Sofosbuvir	3.166	6717883	92210	1.36	9184

Accuracy 120% (Sample):

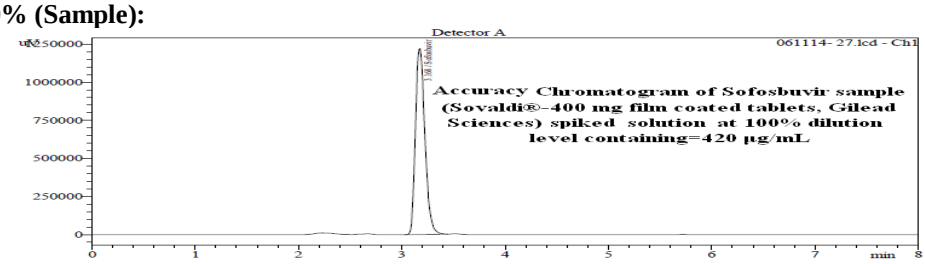

Figure 24: Chromatogram showing Accuracy-120% injection-1

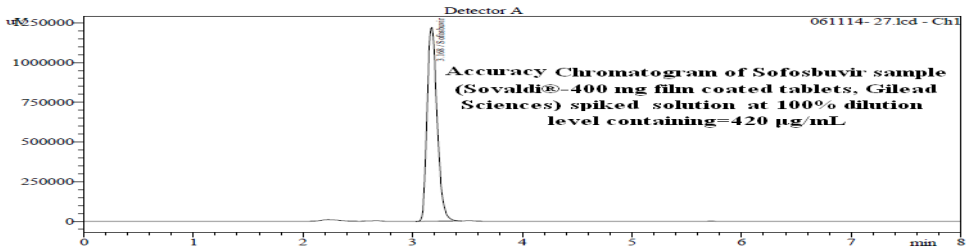

Figure 25: Chromatogram showing Accuracy-120% injection-2

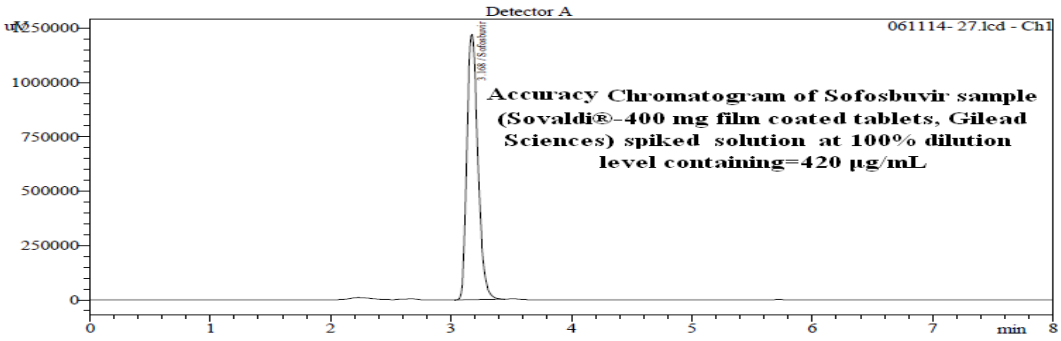

Figure 26: Chromatogram showing Accuracy-120% injection-3

Table 11: Results of Accuracy for Sample injections (Conc.120%)

S.no	Name	Rt (min)	Area	Height	Usp tailing	Usp plate count
1	Sofosbuvir	3.166	7724222	173211	1.36	9463
2	Sofosbuvir	3.169	7770160	173633	1.36	9061
3	Sofosbuvir	3.168	7786289	170368	1.36	9754

Table 12: The accuracy results for Sofosbuvir

%Concentration (at specification Level)	Area	Amount Added (µg/ml)	Amount Found (µg/ml)	% Recovery	Mean Recovery
80%	5681673.0	280	280.5	101%	
100%	6715242	350	349.9	99%	99.6%
120%	7760224	420	418.7	98.9%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).
- The results obtained for recovery at 80%, 100%, 120% are within the limits. Hence method is accurate.

4.4.Precision:

The precision of the method was ascertained separately from the peak area obtained by actual determination of 6 replicas of a fixed amount of drug and formulation. The HPLC systems was set up the described Chromatographic conditions, mentioned as above and follow the system to equilibrate, and then injected the 350 µg/ml concentration of Sofosbuvir standard 6 times and recorded the response (peak area). The proposed method was extended to the pharmaceutical dosage forms by injecting the 350 µg/ml of Sofosbuvir sample with the formulated sample from (Sovaldi®-40mg, Gilead Sciences, film coated tablets)containsSofosbuvirof same concentration 6 times and recorded the response (peak area). The percent relative standard deviation and percent range of error (at 0.05 and 0.01 confidence limits) were calculated and presented in Table.

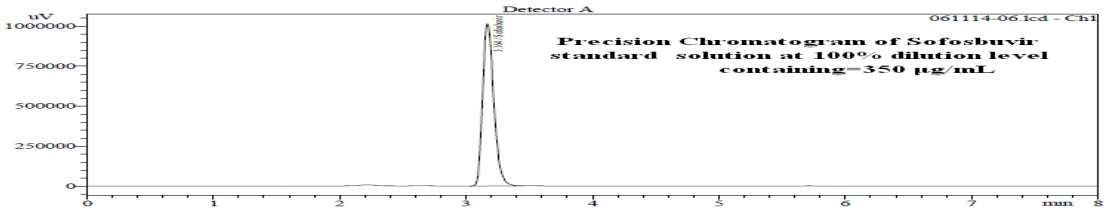

Figure 27: Chromatogram showing precision injection -1

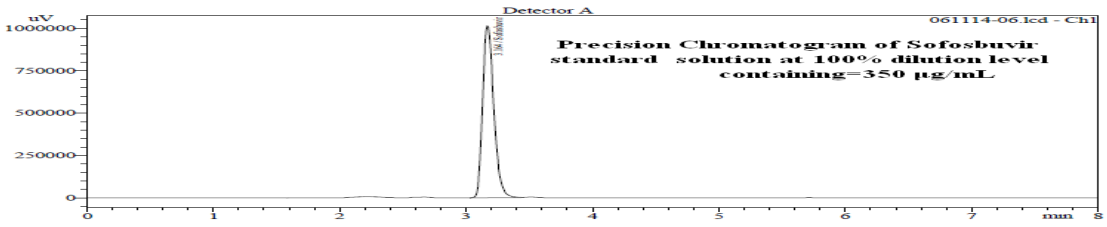

Figure 28: Chromatogram showing precision injection -2

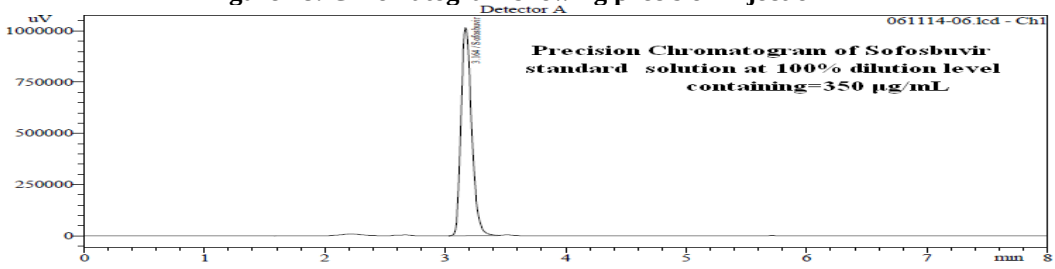

Figure 29: Chromatogram showing precision injection -3

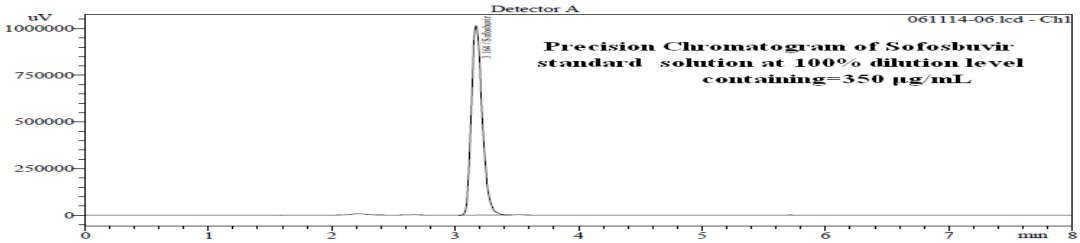

Figure 30: Chromatogram showing precision injection -4

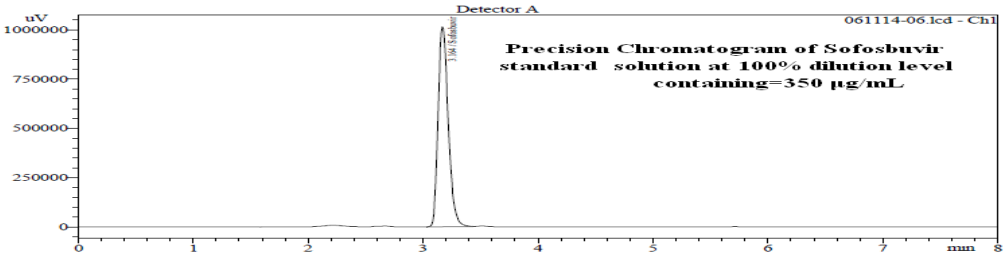

Figure 31: Chromatogram showing precision injection -5

Table 13: Results of precision for Sofosbuvir

Injection No.	Name of the drug	Retention time (min).	Peak Area	Height	Usp plate count
1	Sofosbuvirinjection-1	3.166	6241725	92281	9644
2	Sofosbuvirinjection-2	3.164	6233791	92284	9765
3	Sofosbuvirinjection-3	3.165	6232983	92204	9284
4	Sofosbuvirinjection-4	3.167	6236755	92274	9783
5	Sofosbuvirinjection-5	3.168	6235465	92285	9837
6	Sofosbuvirinjection-6	3.168	6242724	92285	9836
Mean		3.166	6237240		
% RSD.		0.043	0.066		
Std. Deviation		0.001	4089		

INTERMEDIATE PRECISION:

The effect of wide range of intermediates and other additives, usually present in the pharmaceutical dosage forms for batch production, in the determination under optimum conditions were investigated. Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. The commonly used reagents and intermediates present in the Sofosbuvirvial contain powder for injection sample did not interfere with the elution or quantification of the method.

Analysis of Sofosbuvir Film coated tablets:

To find out the suitability of the proposed method for the assay of Sofosbuvir in pharmaceutical dosage forms (Sovaldi® 40 mg, Film coated tablets)the sample solutions from tablets containing Sofosbuvir were analyzed by the proposed method. A homogenized powder of Sovaldi®tabletsofSofosbuvirequivalent to 350mg of the active ingredient was mixed with 50 ml of diluentin 100 ml volumetric flask. The mixture was allowed to stand for 30 minutes with intermittent sonication for complete solubility of the bulk drug, and then filtered through a 0.45 µm membrane filter, followed by addition of mobile phase up 100 ml to obtain a stock solution of 3500µg/mL as the working sample solution. The mixture was allowed to stand for 1 hr with intermittent sonication for complete solubility of the drug, and then filtered through a 0.45 µm membrane filter, followed by addition of mobile phase up 100 ml to obtain a stock solution of 3500µg/mL.The resultant solution was further diluted by taking 5 ml of the stock solution with 50 ml of mobile phase to get the concentration of 350µg/mL.The results are recorded in Table.

Day 1:

Table 14: Results of Intermediate precision for Sofosbuvir

S.no	Peak name	Rt (min)	Area	Height	Usp plate count	Usp tailing
1	Sofosbuvir	3.168	6232590	92274	9184	1.36
2	Sofosbuvir	3.168	6233698	92857	9004	1.36
3	Sofosbuvir	3.169	6234841	92018	9771	1.36
4	Sofosbuvir	3.168	6234773	92271	9448	1.36
5	Sofosbuvir	3.167	6228530	92276	9019	1.36
6	Sofosbuvir	3.168	6229812	92206	9764	1.36
Mean			6232374			
Std.dev			2645			
%RSD			0.042			

Acceptancecriteria:
%RSD of five different sample solutions should not more than

Table 15: Results of Intermediate precision Day 2 for Sofosbuvir

S.no	Peak name	Rt(min)	Area	Height	Usp plate count	Usp tailing
1	Sofosbuvir	3.168	631831	92281	9847	1.36
2	Sofosbuvir	3.168	630696	92277	9164	1.36
3	Sofosbuvir	3.167	633829	92201	9755	1.36
4	Sofosbuvir	3.165	638575	92274	9174	1.36
5	Sofosbuvir	3.166	630228	92265	9575	1.36
6	Sofosbuvir	3.167	631181	92210	9333	1.36
Mean			632723.3			
Std.dev			3129.73			
%RSD			0.04946			

Acceptance criteria:
%RSD of five different sample solutions should not more than 2

4.5.Robustness:
A method is robust if it is unaffected by small changes in operating conditions. To determine the robustness of this method, the experimental conditions were deliberately altered at two different levels and retention time and chromatographic response were evaluated. One factor at a time was changed to study the effect. Variation of the mobile phase flow rate was varied by ±10%) and different column had no significant effect on the retention time and chromatographic response of the method, indicating that the method was robust. When the chromatographic conditions were deliberately altered, system suitability results remained within acceptance limits and selectivity for individual substance was not affected. The results of the study prove the robust nature of the method.

Variation in flow:

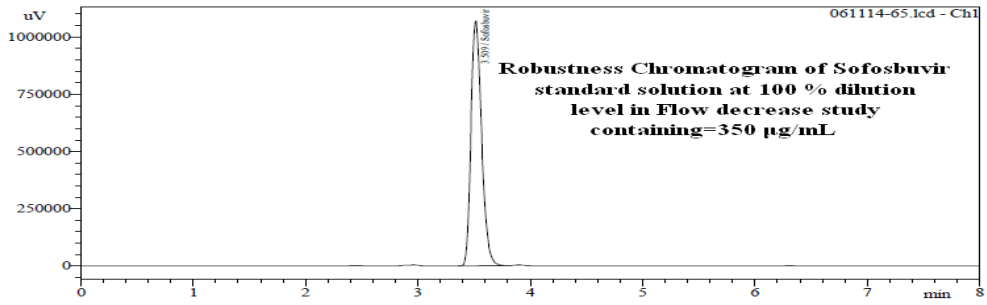

Figure 32: Chromatogram showing less flow of 0.9ml/min in Robustness

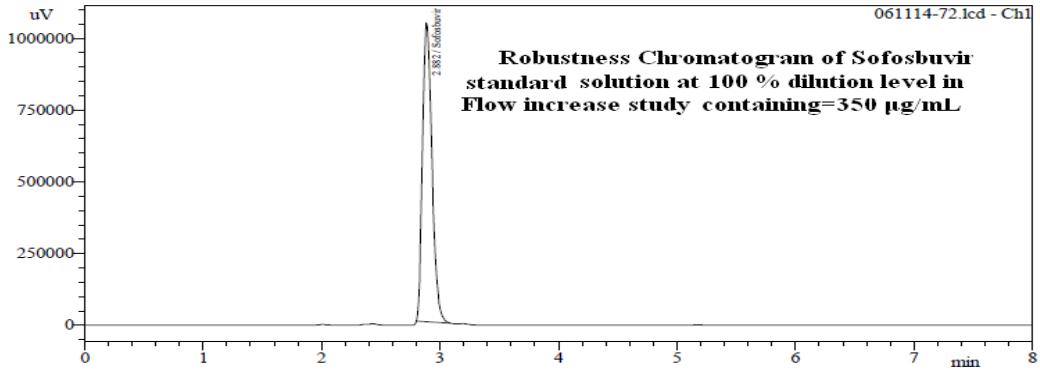

Figure 33: Chromatogram showing more flow of 1.1 ml/min in Robustness

Variation of Mobile Phase Organic Composition

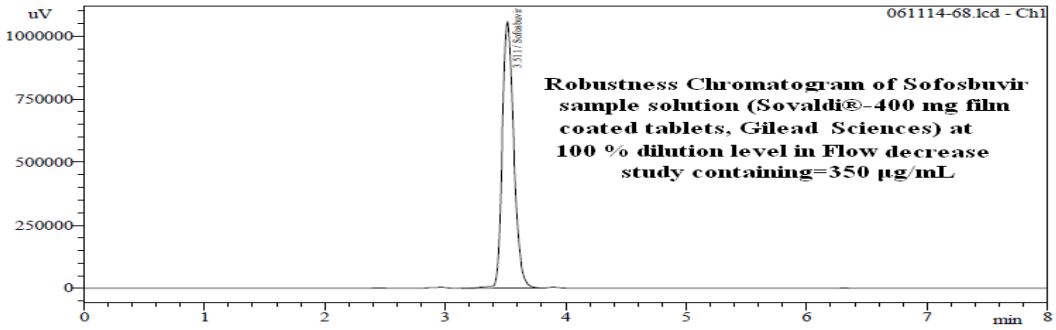

Figure 34: Chromatogram showing Less Organic Composition in Robustness

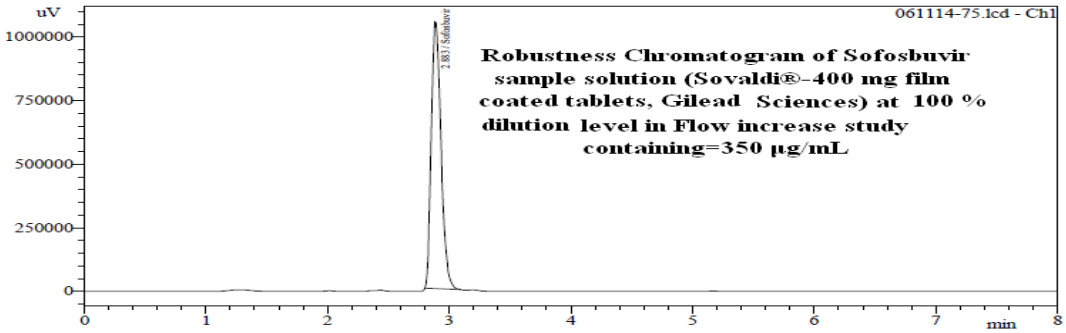

Figure 35: Chromatogram showing More Organic Composition in Robustness

Table 16: Results for Robustness

Parameter used for sample analysis	Peak Area	Retention Time (min)	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	6281207	3.168	9544	1.36
Less Flow rate of 0.9 mL/min	7058410	3.509	8474	1.36
More Flow rate of 1.1 mL/min	5804467	2.882	8575	1.4
Less organic phase	7007582	3.543	7285	1.38
More organic phase	5855502	2.887	7264	1.39

Acceptance criteria:
The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

ASSAY:
The effect of wide range of intermediates and other precursors, generally used in pharmaceutical formulations of Sofosbuvir were investigated under optimized chromatographic conditions. Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. The common excipients present in the pharmaceutical dosage form did not interfere with the elution or quantification of the method. Each Sovaldi®-40 mg Film coated tablet,Gilead Sciences, contains equivalent to Sofosbuvir 40 mg and the tablets include the following inactive ingredients: The inactive ingredients of Sovaldi® Film coated tablet are the following: Tablet Core: lactose monohydrate, microcrystalline cellulose, crospovidone, colloidal silicon dioxide, magnesium stearate. Coating: hypromellose, polyethylene glycol, titanium dioxide, talc, polysorbate 80, FD&C Blue No. 2. Acceptence criteria for specificity, RSD should be less than 2%. The specificity chromatograms are shown in the Figures.

ASSAY(Standard):

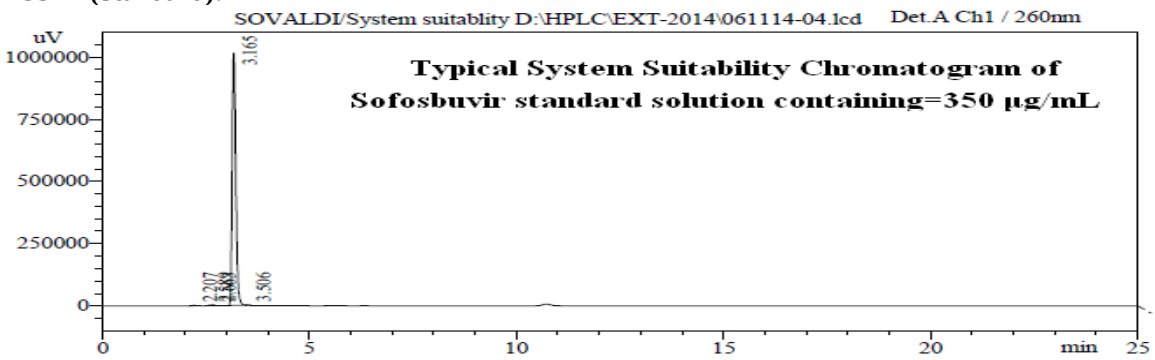

Figure 36: Chromatogram showing Assay of Standard injection -1

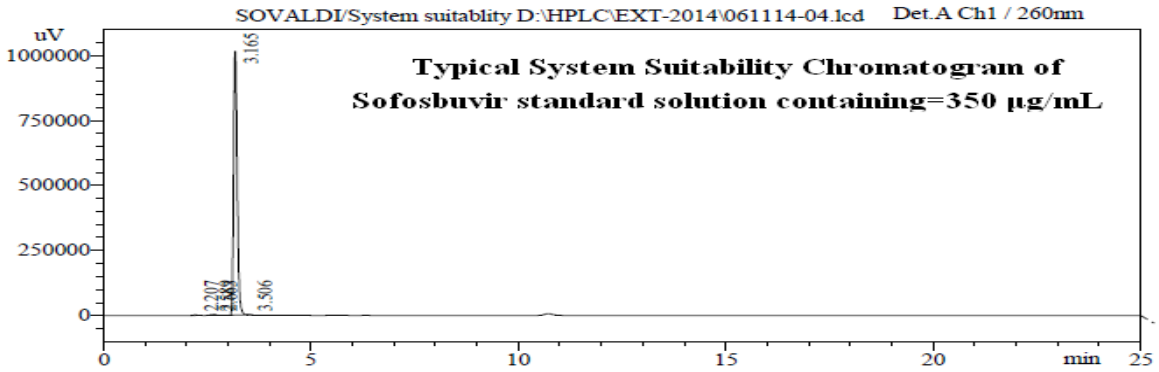

Figure 37 : Chromatogram showing Assay of Standard injection -2

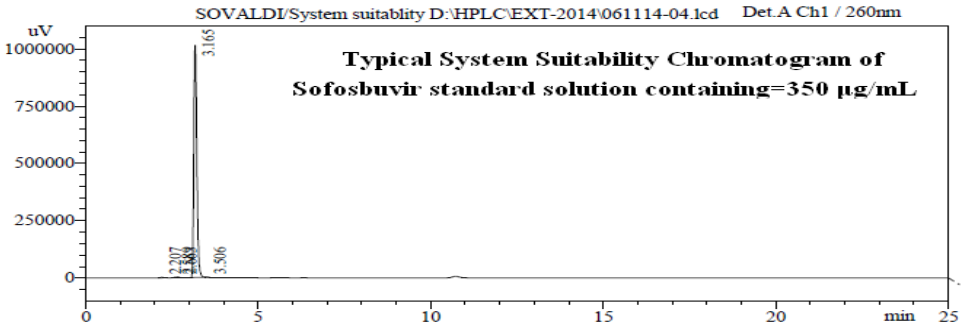

Figure 38 : Chromatogram showing Assay of Standard injection -3

Table 17: Peak results for Assay Standard

S.NO	Name	RT(min)	Area	Height	USP Tailing	USP Plate count
1	sofosbuvir	3.166	631544	92857	1.36	9847
2	sofosbuvir	3.166	631022	92122	1.36	9028
3	sofosbuvir	3.166	631933	92113	1.36	9664

ASSAY (Sample):

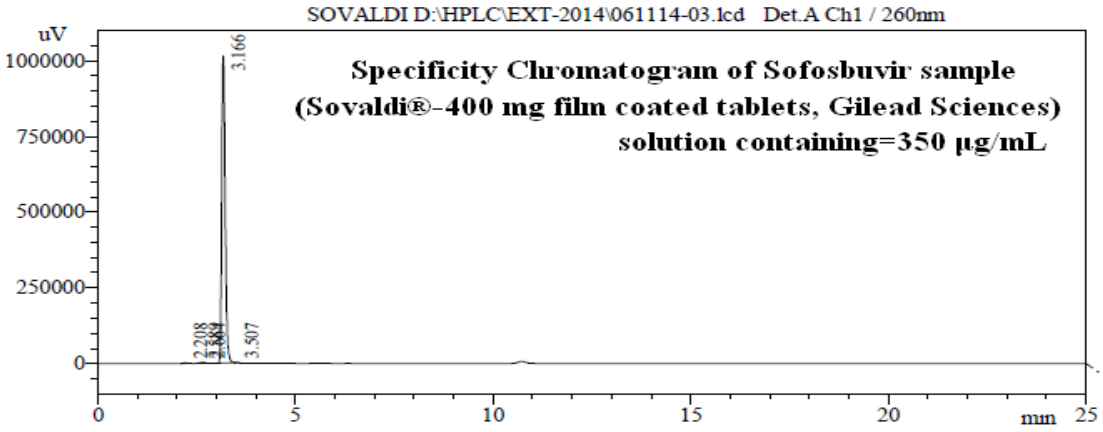

Figure 39 : Chromatogram showing Assay of sample injection-1

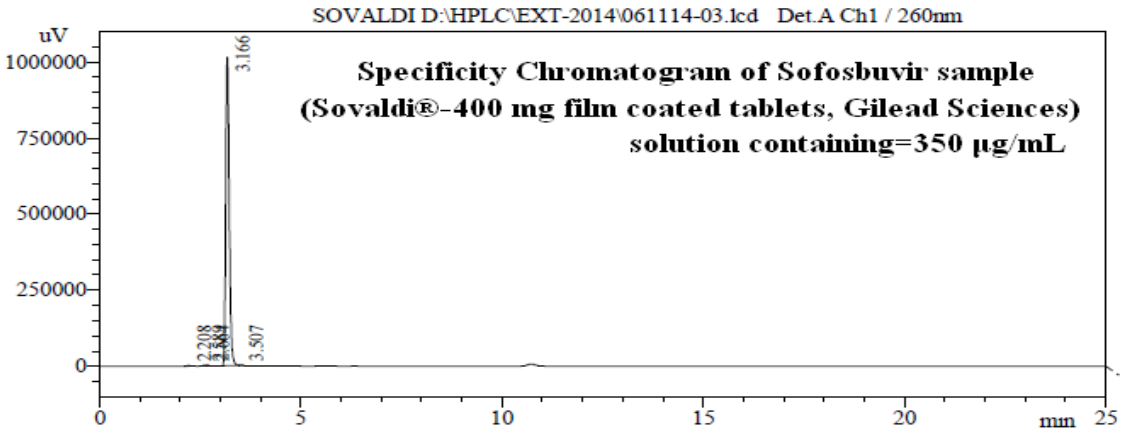

Figure 40: Chromatogram showing Assay of sample injection-2

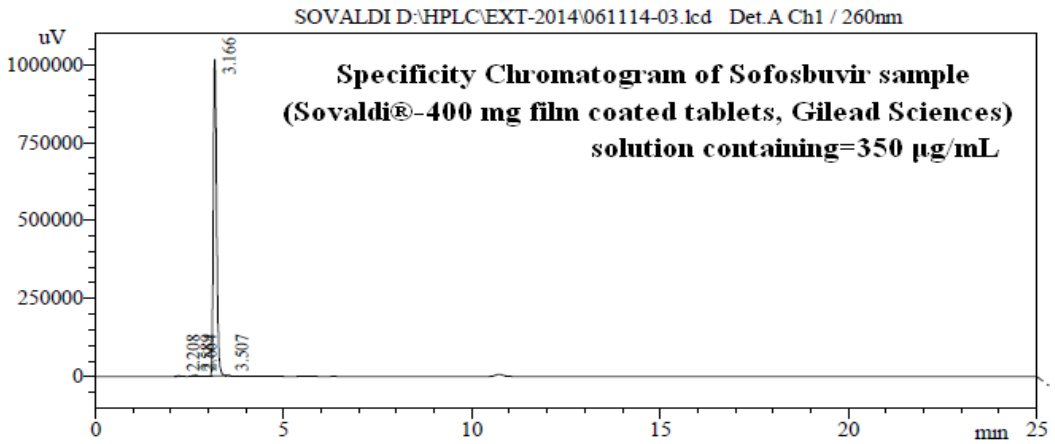

Figure 41 : Chromatogram showing Assay of sample injection-3

Table 18: Peak results for Assay sample

S. No	Name	Rt	Area	Height	USP Tailing	USP Plate Count
1	Sofosbuvir	3.166	631675	92274	1.36	9484
2	Sofosbuvir	3.168	631141	92271	1.36	9081
3	Sofosbuvir	3.165	631019	92281	1.36	9981

Calculation: The amount of Praziqantel and Albendazole present in the formulation by using the formula given below, and results shown in above table:

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

Where,
AS: Average peak area due to standard preparation
AT: Average Peak area due to assay preparation
WS: Weight of Sofosbuvir in mg
WT: Weight of sample in assay preparation
DT: Dilution of assay preparation
AW :Average weight
P: Standard purity
LC: Label claim

%Assay = 631278.3 / 631426.4×10/60×60/0.0198×99.8/100×0.3966/200×100

= 99.9%

Limit of Detection [LOD] and Limit of Quantification [LOQ]:
The detection limit of the method was investigated by injecting standard solutions Sofosbuvir into the HPLC column. By using the signal-to-noise method the peak-to-peak noise around the analyte retention time is measured, and subsequently, the concentration of the analyte that would yield a signal equal to certain value of noise to signal ratio is estimated. A signal-to-noise ratio (S/N) of 3 is generally accepted for estimating LOD and signal-to-noise ratio of 10 is used for estimating LOQ.
This method is commonly applied to analytical methods that exhibit baseline noise. Chromatograms illustrating the LOD are shown in figure 2.10. The limit of detection (LOD) and limit of quantification (LOQ) for Sofosbuvirwere found to be 0.015µg/ml and 0.045 µg/ml respectively.

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 as an exact value.

LOD = 3.3 × σ / s

Where σ = Standard deviation of the response

S = Slope of the calibration curve

Result = 3.3 × 364.7 / 17369.45= 0.07µg/ml

LIMIT OF QUANTITATION FOR SOFOSBUVIR:
The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ = 10 × σ / S

Where σ = Standard deviation of the response

S = Slope of the calibration curve

Result = 10 × 364.73 / 17369.45 = 0.21µg/ml

Performance and Detection Characteristics of Sofosbuvir:

PARAMETES	SOFOSBUVIR
Calibration Range(µg/ml)	35-420 µg/ml
Optimized Wavelength	260 nm
Mobile Phase	Phosphate buffer : Acetonitrile (45:55v/v)
Column	Zodiac C18 Column (250×4.6 ×5µ)
Retention Time	3.166 min
Regression Equation	Y=17369.45x+279381.36
Correlation Coefficient(R^2)	0.999
Precision(%RSD) i) Repeatability	0.066
ii) Intermediate Precision (Day 1)	0.042
Intermediate Precision (Day 2)	0.066
% Recovery	99.9%
LOD(µg/ml)	0.07µg/ml
LOQ(µg/ml)	0.21µg/ml

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