Validated reversed-phase liquid chromatographic method with gradient elution for simultaneous determination of the antiviral agents

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ABSTRACT

A Validated, reversed-phase liquid chromatography (RP-HPLC) method for with gradient elution for simultaneous determination of Sofosbuvir and Daclatasvir in their dosage forms. An accurate, reproducible method was developed and validated. The mobile phase contained a mixture of 90% methanol: 10% water (0.05% OPA). UV detection was performed at a temperature of 30° C., a flow rate of 0.7 ml/min and at a wavelength of 275 nm. The retention times for sofosbuvir and daclatasvir are 3.361 and 5.745 minutes, respectively. This developed and validated method was successfully used for quantitative analysis of commercial dosage forms. The %RSDs for sofosbuvir and daclatasvir were 0.43 and 0.28, respectively. Recoveries were 97.85% and 98.52% for sofosbuvir and daclatasvir, respectively. Three methods were validated according to the International Conference on Harmonization (ICH) guidelines for accuracy, precision, linearity, selectivity, specificity, limits of detection, limits of quantification, robustness and ruggedness.

Keywords: Sofosbuvir, Daclatasvir; RP- HPLC; Validation, Chromatography.

INTRODUCTION

Multi-additives formulations have earned a number of importances nowadays due to higher patient acceptance, increased potency, multiple movements, less side effects, and speedier treatment. There's a plethora of analysis of such formulations without earlier separation. For the estimation of multi-thing formula the instrumental strategies that are normally employed are spectrophotometry, GLC, high overall performance skinny layer chromatography (HPTLC), HPLC and so on. Those strategies are based upon the size of unique and nonspecific physical homes of the materials[1]. The current look at

focuses on the many procedures and parameters involved in an HPLC situation. HPLC technology development is critical for drug discovery, drug improvement, and pharmaceutical products.

It appears to be suitable for routine, pleasant management of research and formulation tests. It focusses specifically on the optimisation of HPLC settings and other critical factors in the process of system enhancement and validation of medicinal compounds.[2]. The analytical technique chosen must be having all the best characteristics and the most crucial being that it must be less time consuming. That is approximately the technique improvement relatively simple project but in case of combined dosage shape the situation is one of a kind because the homes of the one drug may additionally bog down the residences of others. These houses may include solubility, moving of λmax, overlapping of the absorbance, etc. But the numerous sophisticated analytical gadgets at the moment are overcoming those troubles[3].

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

This method is primarily based on the equal technique of separation as classical column chromatography. I.E. Adsorption, partition, ion change and gel permeation but it special from column chromatography, in that cellular section is pumped via the packed column below excessive strain. It's miles the maximum popular method today a few of the extraordinary chromatographic processes. Due to tremendous evolution of Liquid Chromatography (LC) instruments presenting the superior qualitative and quantitative results. The solvent generally flows thru column with the assist of gravity but in HPLC approach the solvent will be compelled under excessive pressures as much as 400 atmospheres so that sample can be separated into exclusive constituent with the help of distinction in relative affinities[4]. In HPLC, pumps can be used to pass pressurized liquid solvent together with the pattern aggregate which is allowed to enter right into a column filled with strong adsorbent material. The interplay of every sample issue will be varies and this causes difference in drift costs of each component an sooner or later results in separation of aspect of column[5]. HPLC relies upon on pumps to skip a pressurized fluid and an instance blend thru a section loaded with adsorbent, prompting the partition of the specimen segments. The dynamic segment of the segment, the adsorbent, is often a granular fabric made from solid debris (eg. Silica, polymers, and many others.) 2 μm to 50 μm in length. The pressurized fluid is normally a mix of solvents (e.G. Water, acetonitrile and/or methanol) and is called ‘cellular phase’. Its business enterprise and temperature performs an crucial component in the partition method through affecting the connections occurring between pattern segments and adsorbent[6]..

Validation of analytical method:[7]

The validation of analytical procedures is based on the following types of analytical procedures:

- To determine the goal of analysis (qualitative / quantitative)
- Select a method for sample preparation (sample clean up: Solid phase extraction, liquid-liquid extraction)

- Choice of the right detector (sensitivity range, destructive/ non-destructive/ responsiveness to analytes).
- Choice of column (diameter, length, particle size, thickness/type of st. phase, RP is adequate to separate low m.wt. neutral or charged organic compounds)
- Choice of injection technique (in GC: split/splitless/on column. In HPLC: Rheodyne anual injector/ autosampler).
- Identification test

Validation[8-9]

Validation of an analytical method is the "**A documented programme, which provides a high degree of assurance that a specific process will consistently produce, a product meeting its pre-determined specifications and quality attributes**".

The developed method should be validated for its,

1. Specificity

Specificity is the ability of a method to discriminate between the intended analyte(s) and other components in the sample. Specificity of the HPLC method is demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients.

2. Accuracy

Accuracy is the closeness in agreement of the accepted true value or a reference value to the actual result obtained. Accuracy studies are usually evaluated by determining the recovery of a spiked sample of the analyte into the matrix of the sample (a placebo).

3. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements which is obtained from multiple sampling of the same homogeneous sample under the prescribed conditions .Method precision is a measure of the ability of the method to generate reproducible results. The precision of a method is evaluated for repeatability, intermediate precision, and reproducibility.

- **Repeatability** is a measure of the ability of the method to generate similar results for multiple preparations of the same homogeneous sample by one analyst using the same instrument in a short time duration (e.g., on the same day).
- **Intermediate precision, synonymous with the term "ruggedness,"** is a measure of the variability of method results where samples are tested and compared using different analysts, different equipment, and on different days, etc. It is a measure of the intra-laboratory variability and is a measure of the precision that can be expected within a laboratory.
- **Reproducibility** is the precision obtained when samples are prepared and compared between different testing sites. Method reproducibility is often assessed during collaborative studies at the time of method transfer (e.g., from a research facility to quality control of a manufacturing plant).

4. Limit of Detection

The limit of detection is the smallest amount or concentration of analyte that can be detected. There are a number of ways for the calculation of LOD. The simplest method to calculate LOD is to determine the amount (or concentration) of an analyte that yields a peak height with a signal-to-noise ratio (S/N) of 3.

5. Limit of Quantitation

The limit of quantitation is the lowest level that an analyte can be quantitated with some degree of certainty (e.g., with a precision of ±5%).The simplest method for calculating LOQ is to determine the amount (or concentration) of an analyte that yields a peak with a signal-to-noise ratio of 10. Thus, LOQ is roughly equal to 3 times of LOD.

6. Range

The range of an analytical method is the interval between the upper and lower analytical concentration of a sample that has been demonstrated to show acceptable levels of accuracy, precision, and linearity.

7. Robustness

The robustness is a measure of the performance of a method when small, deliberate changes are made to the specified method parameters. The intent of robustness validation is to identify critical parameters for the successful implementation of the method.

8. Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, analyst instruments and lots of reagents, elapsed assay times, assay temperature or days. Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst.

9. **Linearity**

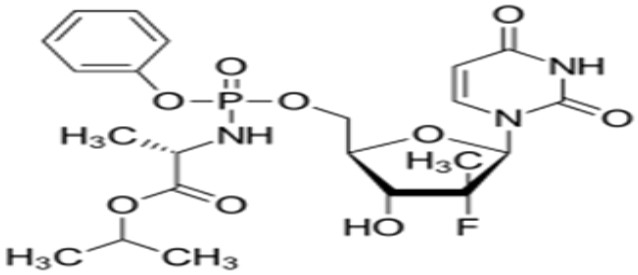
The linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range, using the relationship between detector response (peak area or height) and sample concentration (or amount).

DRUG PROFILE

SOFOSBUVIR[10]

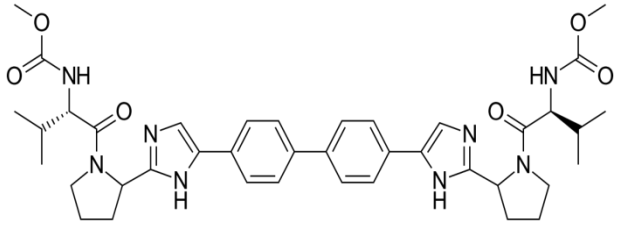
Table 1: physiochemical data for sofosbuvir

Drug name	SOFOSBUVIR
IUPAC Name	S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl)methoxy)-(phenoxy)phosphorylamino)propanoate
State	Solid
Molecular formula	$C_{27}H_{28}N_2O_7$
Molecular Weight	529.453 g/mol

Melting point	76-82 °C
Description	White to off white crystalline solid
Solubility	soluble in water,methanol,acetonitrile
Pka(stronger acidic)	9.7
Pka(stronger basic)	-3.9
Therapeutic category	Antiviral
Molecular Structure	

DACLATASVIR[11]

Table 2: physiochemical data of daclatasvir

Drug name	Daclatasvir
IUPAC Name	methyl N-[(2S)-1-[(2S)-2-[5-(4'-{2-[(2S)-1-[(2S)-2-[(methoxycarbonyl)amino]-3-methylbutanoyl]pyrrolidin-2-yl]-1H-imidazol-5-yl}-[1,1'-biphenyl]-4-yl)-1H-imidazol-2-yl]pyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl]carbamate
State	Solid
Molecular formula	$C_{40}H_{50}N_8O_6$
Molecular Weight	738.89 g/mol
Melting point	234°C
Description	white to yellow crystalline non-hygroscopic podwer
Water Solubility	soluble in water,Methanol,acetronitle
Pka(stronger acidic)	11.15
Pka(stronger basic)	6.09
Therapeutic category	Antiviral
Molecular Structure:	

MATERIAL AND INSTRUMENTS

Materials:

The drugs used for the present investigation were obtained from Hetero Pharmaceuticals Pvt. Ltd. as gift sample.

Details of Pure drug:

Table 3: Description of Drugs

Drug	Supplied by	Quantity	Purity (Assay)
SOFOSBUVIR	Hetero Pharma	10 g	99.8 % w/w
DALACTASVIR	Hetero Pharma	10 g	99.02% w/w

Reagents and chemicals:

All reagents and chemicals used were of AR grade and HPLC grade.

1. Methanol (HPLC grade).
2. Distilled Water (HPLC grade).
3. Ortho Phosphoric Acid (HPLC grade).

Instruments

Table 4: Instruments used for the experiment

Sr No.	Instrument	Make	Model
1	UV-Visible Spectrophotometer	Thermo Electron Shimadzu	Double beam carry-07 Bio UV 1601
2	HPLC	Youglin	UV Detector
3	pH Meter	Equip-tronich	Eq-614A
4	Balance	Citizen	CY-104(Micro Analytical Balance)
5	Ultrasonicator	Meta-Lab	1.5L 50

EXPERIMENTAL WORK

RP-HPLC method development and validation for Sofosbuvir and Daclatasvir.

1. Determination of λ max of Sofosbuvir and Daclatasvir:

The stock standard Solution of Sofosbuvir and Daclatasvir are prepared by dissolving 400mg and 60mg of each drug in 10ml Methanol. The Stock Standard Solutions were further diluted with Methanol to obtain a Concentration of 40000µg/ml and 6000µg/ml Sofosbuvir and Daclatasvir respectively. The λmax was determined on Shimadzu UV-Visible Spectrophotometer (model UV-730D) in the range of 200-400nm using Methanol as a blank. The Solution of mixture exhibited maxima at about 275nm.

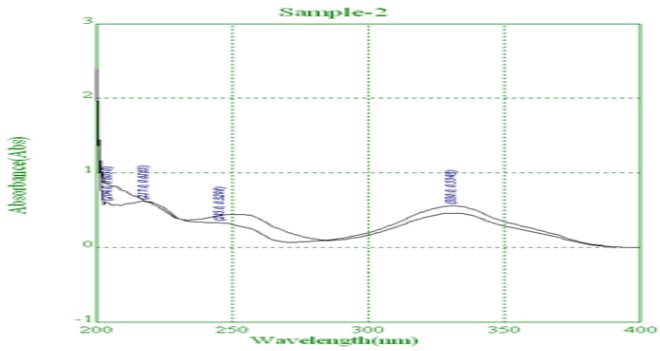


Figure 1: Overlain Spectra of Sofosbuvir and Daclatasvir

2. Selection of mobile Phase:

a) Preparation of Standard Solutions:

Sofosbuvir and Daclatasvir Standard Solution:

Accurately weighted quantity 400mg and 60mg of Sofosbuvir and Daclatasvir was dissolved in Methanol Volume was made up to 10ml mark to get final concentration of about 40000µg/ml of Sofosbuvir and 6000µg/ml Daclatasvir.

b) Procedure:

The optimization of HPLC method were done for the selection of proper mobile phase for method development Pure drug products were injected and run in different solvent systems. In this, different trials are taken with different ratio of mobile phase. For trials methanol and water at different flow rate and pH were used. Different combinations of mobile phases were tried for selections of proper mobile phase are given below table.

Table 5: Result of different trials

Fig. No.	Column used	Mobile phase, Flow Rate and Wavelength	Inj. Vol.	Observation	Conclusion
1	C_{18}(COSMOSIL)(250 ×4.6mm, 5.0µ)	80%Methanol: 20% Water (0.1 % OPA) 275 nm, Flow rate 0.7ml.	20 µl	Well resolved peaks were not obtained	Hence rejected
2	C_{18}(COSMOSIL)(250 ×4.6mm, 5.0µ)	70%Methanol:30% Water (0.1% OPA) 275 nm, Flow rate 0.7ml	20 µl	peaks were not obtained	Hence rejected
3	C_{18}(COSMOSIL)(250 ×4.6mm, 5.0µ)	90%Methanol :10% Water (0.05%OPA)-275 nm, Flow rate 0.7ml	20 µl	Well resolved peaks were obtained	Hence selected

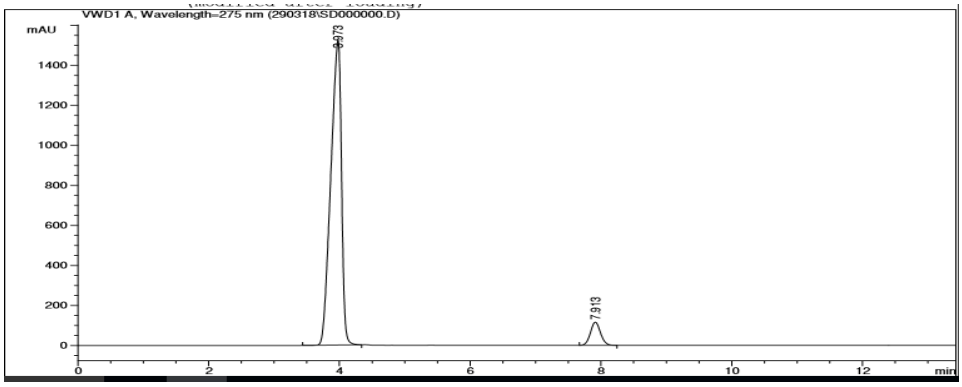


Figure 2: Chromatogram of trail 1 using 80% Methanol and 20% Water (0.1% OPA) Flow rate 0.7ml at 275nm.

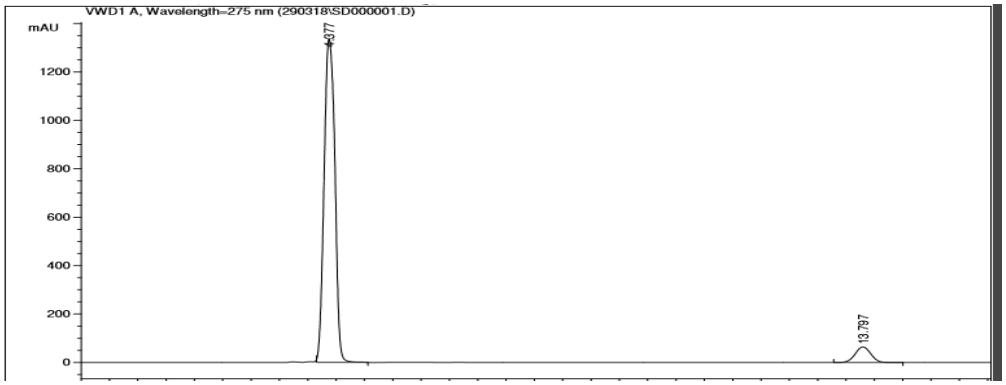


Figure 3: Chromatogram of trail 2 using 70% Methanol and 30% Water (0.1 % OPA) Flow rate 0.7ml at 275 nm

From various mobile phases tried, mobile phase containing, Methanol: Water (90:10) (0.1%OPA)) was selected, since it gives sharp reproducible retention time for SOFOSBUVIR AND DACLATASVIR is 3.361 min and 5.745 min.

Chromatographic Parameter:

Column	:	C_{18} (COSMOSIL) 4.6 ×250
Flow Rate	:	0.7ml/min
Wavelength	:	275nm
Injection Volume	:	20µl
Column Oven Temperature	:	Ambient
Run Time	:	16 minutes
Mobile Phase	:	Water (0.1%OPA) and Methanol (90:10)

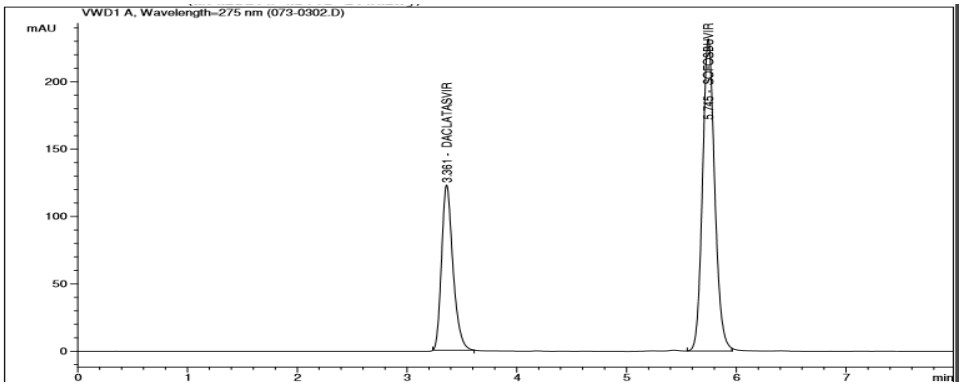


Figure 4: Chromatogram obtained by using Methanol: Water (90:10) as mobile phases

Preparation of Calibration Curves sings HPLC:

Standard Stock Solution:

Accurately weighed Sofosbuvir 400mg and Daclatasvir 60mg dissolved in 10ml Methanol. This Solution was used as Standard Stock Solution. Thus Stock Solution holds Sofosbuvir 40000µg/ml and Daclatasvir 6000µg/ml. The Linearity of Analytical procedure is its ability to obtained test result which are directly proportional to the concentration of analyte in the sample. The range of an Analytical Procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, linearity. The dilute portions of Standard Stock Solutions of Sofosbuvir 400mg and Daclatasvir 60mg was diluted appropriately with mobile phase to get series of concentration from for both drugs. The absorbance of this drug was measured at 275 nm respectively and calibration curve was plotted as concentration versus absorbance.

Procedure:

The mobile phase was allowed to equilibrate with the Stationary phase until steady baseline was obtained. The series of concentration Sofosbuvir and Daclatasvir Standard Solutions were injected and peak area as recorded

FOR SOFOSBUVIR

Table No.6.Observations of Standard Calibration Curve of Sofosbuvir

Sr No.	Conc.	Area-I	Area-II	Mean	SD	%RSD
1	30	592.63	596.25	594.44	2.56	0.43
2	60	1178.47	1169.87	1174.17	6.08	0.52
3	90	1757.27	1789.08	1773.175	22.49	1.27
4	120	2404.53	2389.5	2397.015	10.63	0.44
5	150	2936.61	2932.76	2934.685	2.72	0.09

FOR DACLATASVIR:

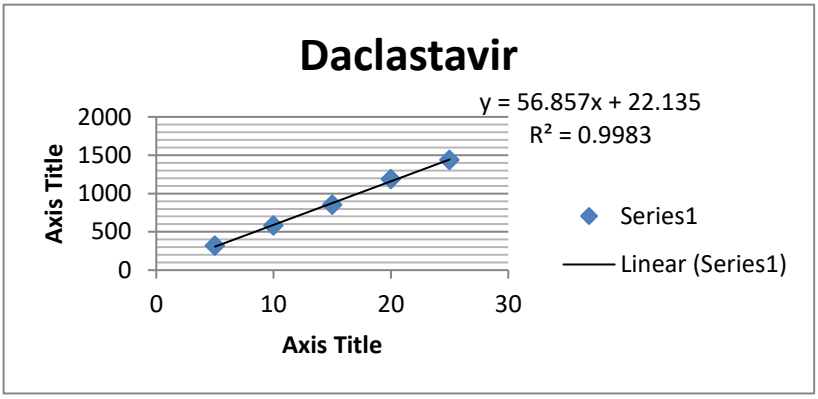


Figure 5: Various Concentration and Average Area of Daclatasvir

Table 7: Observations of Standard Calibration Curve of Daclatasvir

Sr.No.	Conc.	Area-I	Area-II	Mean	SD	%RSD
1	5	315.39	316.64	316.01	0.88	0.28
2	10	586.38	578.86	582.62	5.32	0.91
3	15	854.39	854.12	854.255	0.19	0.02
4	20	1187.22	1186.01	1186.615	0.86	0.07
5	25	1438.47	1432.4	1435.435	4.29	0.30

System Suitability test:

System Suitability is a Pharmacopoeial reuirement and is used to verify,whether the resolution and reproducibility of the Chromatographic system are adequate for analysis to be done. The tests were performed by collecting data fom five replicate injections of standards.

Preparation of standard stock solution:

Sofosbuvir Standard Solution:

Accurately weighed Sofosbuvir 400mg was dissolved in mobile phase and volume was make upto 10ml Methanol. The stock Solution was diluted further with mobile phase to get final concentration of about 40000µg/ml of Sofosbuvir.

Daclatasvir Standard Solution:

Accurately weighed Daclatasvir 60mg was dissolved in mobile phase and volume was make upto 10ml Methanol. The stock Solution was diluted further with mobile phase to get final concentration of about 6000µg/ml of Daclatasvir.

B) Procedure:

Filtered mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. A 20µl std. drug solution was injected which was made in five replicates and system suitability parameters were recorded

Table 8: Showing result of System Suitability Parameters

Sr.No	Peak area		Retention Time		Asymmetry		No .of theoretical Plates		Resolution
	DAC	SOFO	DAC	SOFO	DAC	SOFO	DAC	SOFO	
1.	582.862	1169.969	3.392	5.597	0.71	0.84	5600	13531	12.49
2	582.46	1172.46	3.293	5.595	0.71	0.85	5603	13918	12.57
3	583.321	1170.63	3.293	5.595	0.71	0.85	5784	13918	12.66
MEAN	582.881	1171.01	3.326	5.59	0.71	0.84	5662.33	13789.0	12.57
± S.D	0.43081	1.290	0.057	0.001	0.0	0.005	105.37	223.43	0.085
%RSD	0.7	0.11	1.72	0.02	0	0.68	1.86	1.62	0.68

Application of Proposed Method for estimation of Sofosbuvir and Daclatasvir Laboratory mixture:

Preparation of laboratory mixture (standard):

Accurately weighed quantity of Sofosbuvir 400mg was transferred to 10ml Volumetric flask Shaken Vigorously for five minutes &Volume was made up to mark with mobile phase. And Accurately weighed quantity of Daclatasvir 60mg was transferred to 10ml Volumetic flask,Shaken vigorously for five minutes and volume was made up to mark with mobile phase. The standard solution of Sofosbuvir and Daclatasvir were mixed and diluted with mobile phase properly to obtained laboratory mixture containing a concentration 10µg/ml of Sofosbuvir and 20µg/ml of Daclatsvir.

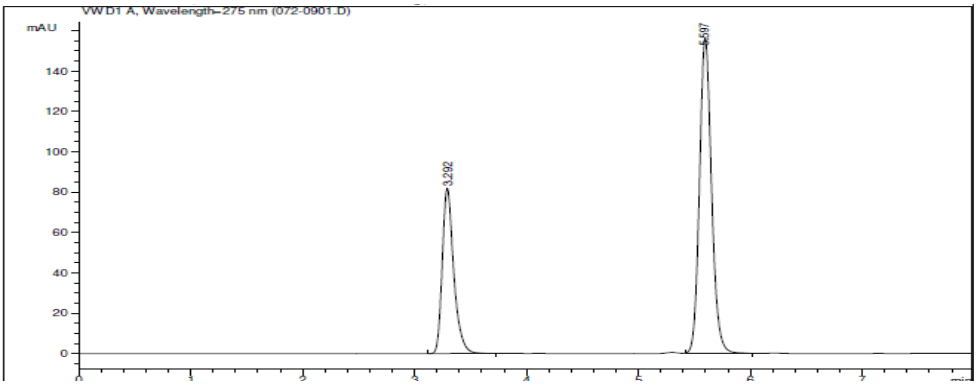


Figure 7: Estimation of sofosbuvir and Daclatasvir in Laboratory mixture

Preparation of laboratory mixture (sample):

Five different laboratory mixtures of Sofosbuvir and Daclatasvir are prepared by appropriately weighing the quantities of drug samples so as to get the concentration of 400µg/ml of Sofosbuvir and 60µg/ml of Daclatasuvir. The peak area of standard laboratory mixture and sample laboratory mixture compared to obtained the concentration.

Table 9: Result and Statistical data for estimation of Sofosbuvir and Daclatasvir in Laboratory mixture

Sr.no	Conc of sample		Peak area of sample		Amount found		% label claim	
	SOFO	DACL	SOFO	DACL	SOFO	DACL	SOFO	DAC
1	120	20	2356.69	1184.82	119.62	20.45	99.68	102.25
2	120	20	2332.71	1171.39	118.40	20.21	98.67	101.05
Mean					119.01	20.33	99.17	101.65
Standard deviation					0.862	0.169	0.71	0.84
%Relative Standard deviation					0.02	0.83	0.72	0.83

Application of Proposed Method for estimation of Sofosbuvir and Daclatasvir in formulation:

Standard Stock Solution:

Accurately weighed quantity of Sofosbuvir 400mg was transferred to 10ml Volumetric flask**.**Shaken Vigorously for five minutes &Volume was made up to mark with mobile phase. And Accurately weighed quantity of Daclatasvir 60mg was transferred to 10ml Volumetric flask,Shaken vigorously for five minutes and volume was made up to mark with mobile phase. The standard solution of Sofosbuvir and Daclatasvir were mixed and diluted with mobile phase properly to obtained laboratory mixture containing a concentration 400µg/ml of Sofosbuvir and 60µg/ml of Daclatasvir.

Sample Solution Preparation:

Five different tablet mixtures of Sofosbuvir and Daclatasvir are prepared by appropriately weighing the quantities of drug samples so as to get the concentration of 400µg/ml of Sofosbuvir and 60 µg/ml of Daclatasvir.

Procedure:

Equal volume (20µg/ml) of Standard and sample solution were injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response i.e. Peak area of major peaks were measured. The content of sofosbuvir and Daclatasvir was calculated by comparing a sample peak with that of standard.

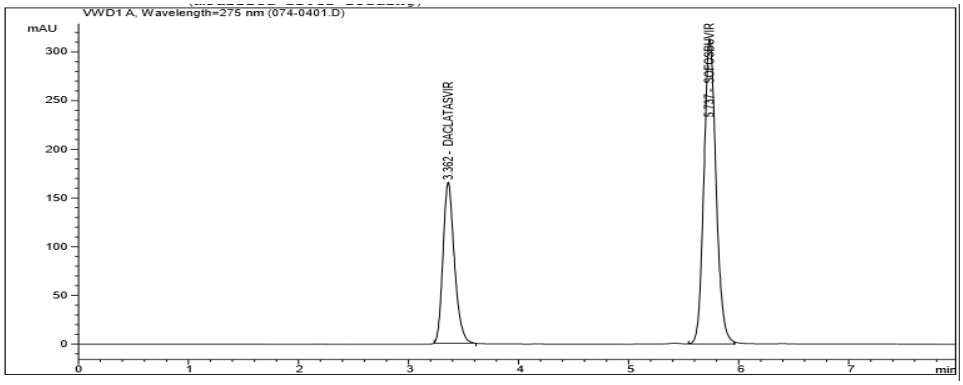


Figure 8: Chromatogram obtained by tablet formulation of Sofosbuvir and Daclatasvir

Table 10: Results and statistical data for estimation of Sofosbuvir and Daclatasvir in marketed formulation.

Sr. no	Conc.of sample		Peak area of sample		Amount found		% label claim	
	SOFO	DACL	SOFO	DACL	SOFO	DACL	SOFO	DACL
1	30	5	592.632	315.399	29.94	5.15	99.8	103.16
2	30	5	596.254	316.645	30.12	5.180	100.4	103.60
Mean					30.03	5.165	100.1	103.38
Standard deviation					0.127	0.021	0.424	0.31
%Relative Standard deviation					0.42	0.41	0.42	0.30

VALIDATION:

Validation Parameters:

A) Accuracy:

Recovery studies were performed to validate the accuracy of developed method. To pre analysed tablet solution, a definite concentration of standard drug (80%, 100% and 120%) was added and then its recovery was analyzed (Table No.13). Statistical validation of recovery studies shown in (Table No.14).

Table 11: Recovery studies of Sofosbuvir and daclatasvir

Level of Recovery (%)	80		100		120	
	SOFO	DACL	SOFO	DACL	SOFO	DACL
Amount present (mg)	60 60	5 5	60 60	5 5	60 60	5 5
Amount of Std. Added (mg)	48 48	4 4	60 60	5 5	72 72	6 6
Amount Recovered (mg)	46.75 46.61	3.97 4.02	58.71 58.23	4.92 5.02	71.26 70.41	5.83 5.98

% Recovery	**97.39** **97.11**	**99.42** **100.54**	**97.85** **97.05**	**98.52** **100.47**	**98.97** **97.80**	**97.16** **99.73**

Table 12: Statistical Validation of Recovery Studies

Level of Recovery (%)	Drug	Mean Recovery	Standard Deviation	% RSD
80	**Sofosbuvir**	**97.39**	**0.20**	**0.20**
	Daclatasvir	**99.98**	**0.79**	**0.79**
100	**Sofosbuvir**	**97.45**	**0.57**	**0.58**
	Daclatasvir	**99.50**	**1.38**	**1.39**
120	**Sofosbuvir**	**101.58**	**0.83**	**0.81**
	Daclatasvir	**101.58**	**1.82**	**1.79**

B) Precision:-

The method was established by analyzing various replicates standards of Sofosbuvir and daclatasvir. All the solution was analyzed thrice in order to record any intra-day & inter-day variation in the result. The results obtained for intraday are shown in Table No 17 & 18 the result obtained for interday variations are shown in the Table No15 &16 respectively.

a) Interday Sofosbuvir

Table 13: Inter-day Precision study of Sofosbuvir

Sr No.	Conc	Area I	Area II	Mean	Amt Found	% Amt Found	SD	%RSD
1	30	609.4679	607.2564	608.36	30.74	102.47	0.96	0.16
2	90	1750.7894	1751.2145	1751.00	88.83	98.70	0.30	0.02
3	150	2930.66	2931.21	2930.94	148.81	99.21	0.39	0.01

b) Interday Daclatasvir

Table 14: Inter-day Precision study of Daclatasvir

Sr No.	Conc.	Area I	Area II	Mean	Amt Found	% Amt Found	SD	%RSD
1	5	308.9612	317.1236	312.04	5.09	101.80	5.77	1.85
2	15	854.2653	855.4621	854.86	14.64	97.60	0.85	0.10
3	25	1440.42	1432.56	1436.49	24.87	99.48	5.56	0.39

c) Intra-day Sofosbuvir

Table 15: Intra-day Precision study of Sofosbuvir

Sr No.	Conc	Area I	Area II	Mean	Amt Found	% Amt Found	SD	%RSD
1	30	608.74	600.12	604.43	30.54	101.80	6.10	1.01

2	90	1752.8	1747.98	1750.39	89.17	99.07	3.41	0.19
3	150	2922.73	2926.98	2924.86	148.50	99.00	3.01	0.10

d) Intra-day Daclatasvir

Table No. 16. Intra-day precision study of Daclatasvir

Sr No.	Conc	Area I	Area II	Mean	Amt Found	% Amt Found	SD	%RSD
1	5	309.25	309.22	309.24	5.05	101.00	0.02	0.01
2	15	861.41	860.4	860.90	14.75	98.36	0.71	0.08
3	25	1439.52	1444.13	1441.83	24.97	99.88	3.26	0.23

C) Specificity:

Specificity was measured as ability of the proposed method to obtained well separated peaks for Sofosbuvir & Daclatasvir without any interference from component of matrix.

Mean retention time for-

Sofosbuvir: 5.749

Daclatasvir: 3.349

The values obtained were very close to that in standard laboratory mixture indicates no interfearence from components of matrix.

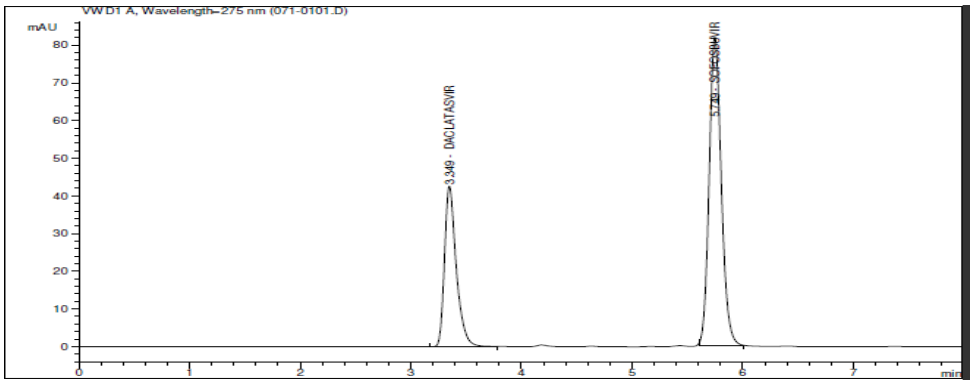


Figure No. 11: Chromatogram obtained by tablet formulation of Sofosbuvir and Daclatasvir

D) Linearity and Range:

From the stock standard solution, aliquots portions were transferred into a series of 10.0 mL volumetric flasks and diluted up to the mark with mobile phase to obtain final concentration in the range of 30-150µg/ml for SOFO and 5-25 µg/ml for DAC.

A constant volume of 20.0 µL of each sample was injected and calibration curve was constructed by plotting the peak area *versus* the drug concentration**.** The observations are shown in Table

Table no. 17. Linearity study of Sofosbuvir

Sr No.	Conc.	Area-I	Area-II	Mean	SD	%RSD

1	30	592.63	596.25	594.44	2.56	0.43
2	60	1178.47	1169.87	1174.17	6.08	0.52
3	90	1757.27	1789.08	1773.175	22.49	1.27
4	120	2404.53	2389.5	2397.015	10.63	0.44
5	150	2936.61	2932.76	2934.685	2.72	0.09

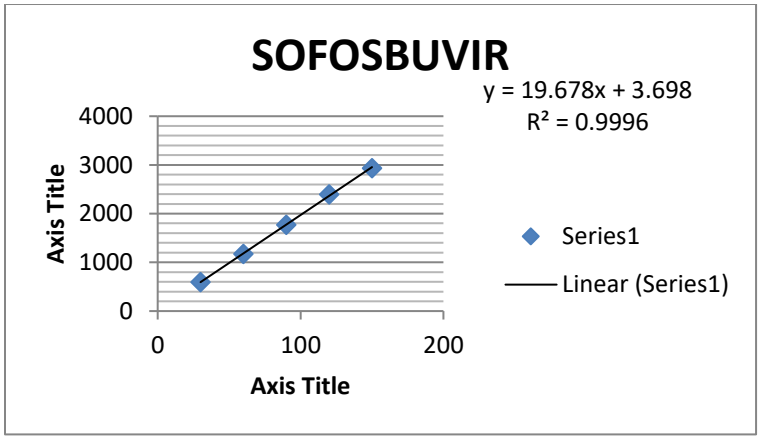


Figure No.12: Observations of Linearity Study of Sofosbuvir

Table.18: Regression equation data for Sofosbuvir

Regression Equation Data y = 19.67x + 3.698	
Slope(m)	19.67
Intercept(c)	3.698
Correlation Coefficient	0.999

Table No. 19: Linearity study of Daclatasvir

Sr No.	**Conc**	**Area-I**	**Area-II**	**Mean**	**SD**	**%RSD**
1	5	315.39	316.64	316.01	0.88	0.28
2	10	586.38	578.86	582.62	5.32	0.91
3	15	854.39	854.12	854.255	0.19	0.02
4	20	1187.22	1186.01	1186.615	0.86	0.07
5	25	1438.47	1432.4	1435.435	4.29	0.30

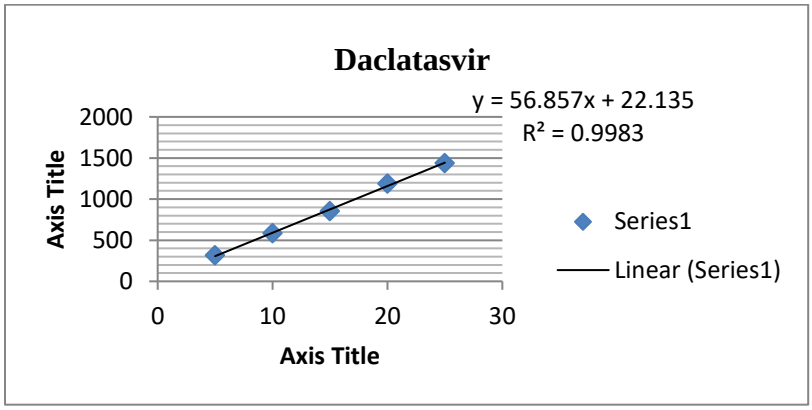


Fig No. 20: Observations of Linearity Study of Daclatasvir

Table No. 23: Regression equation data for Daclatasvir

Regression Equation Data y = 56.857x +22.135	
Slope(m)	56.857
Intercept(c)	22.135
Correlation Coefficient	0.998

E) Robustness:

The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate on retention time and tailing factor of drug peak was studied.

The mobile phase composition was changed in ± 1 ml proportion and the flow rate was varied by ± 0.1 ml min^{-1}, of optimized chromatographic condition. The results of robustness studies are shown in Table. System suitability parameters were also found satisfactory; hence the analytical method would be concluded.

Table No. 21: Result of Robustness Study of Sofosbuvir

Parameters	**Conc.**	**Amount of detected(mean ±SD)**	**%RSD**
Mobile phase composition(89+11)	60	1040±3.17	0.31
Mobile phase composition(91+09)	60	2297.18 ±0.95	0.04
Wavelength change 274nm	60	1284.1±2.62	0.20
Wavelength Change 276 nm	60	1047.41 ± 4.00	0.38
Flow rate change(0.6ml)	60	1372.84±3.37	0.25

Flow rate change(0.8ml)	60	1005.30±7.10	0.71

Table No. 22: Result of Robustness Study of Daclatasvir

Parameters	**Conc.**	**Amount of detected(mean ±SD)**	**%RSD**
Mobile phase composition(89+11)	10	625.4±2.28	0.36
Mobile phase composition(91+09)	10	1112.28±1.37	0.12
Wavelength change 274nm	10	556.8±2.69	0.48
Wavelength Change 276nm	10	622.79±3.71	0.59
Flow rate change(0.6ml)	10	670.32±0.71	0.11
Flow rate change(0.8ml)	10	495.38±4.26	0.86

RESULT AND DISCUSSION

Development and validation of RP-HPLC for the simultaneous estimation of Sofosbuvir and daclatasvir in bulk and combined tablet dosage form. RP-HPLC method was developed for simultaneous estimation of Sofosbuvir and Daclatasvir in tablet dosage form. The separation was achieved by C_{18} (COSMOSIL) column of (4.6×250 mm) with particle size packing 5 µm and Methanol: 0.1% OPA Water(90:10) as mobile phase at a flow rate of 0.7 ml/min. The detection was carried out at 275 nm. The retention time of was found to be 3.697 min and 6.089 min respectively. After establishing the chromatographic conditions, analysis of tablet formulation was done.

Validation:

1. System suitability test:

System suitability was performed to verify, whether the resolution and reproducibility of the chromatographic system are adequate. (Table No. 11, 12)

2. Accuracy :

Accuracy of method is ascertained by recovery studies performed at different levels of concentrations (80%, 100% and 120%). The % recovery was found to be within 99-101% (Table No. 14, 15)

3. Precision:

Precision studies were carried out using parameter like intra-day and inter-day precision, the study showed that the results were within acceptance limit.i.e. %RSD below 2.0 indicating reproducibility of method. Results are shown in Table (16, 17, 18, and 19).

4. Specificity:

Is the ability to assess unequivocally the analyte in the presence of impurities, degradants, matrix etc. It is evaluated by injecting the blank and the control sample solution prepared as per the proposed method to check for the interference if any peak at the retention time of Sofosbuvir and Daclatasvir. Thus no interference was found at the Retention time of Sofosbuvir and daclatasvir which is 5.74 & 3.34 respectively.

5. Linearity:

Linearity of Sofosbuvir was observed in the range of 30-150 µg/ml and Daclatasvir was observed in the range of 5-25 µg/ml Detection of wavelength used was 275nm. (Table. No. 20, 21, 22, 23) The calibration curve yielded correlation coefficient (r^2) 0.999 & 0.998 for Sofosbuvir and Daclatasvir respectively.

6. Robustness:

To evaluate the robustness of the method, the parameters selected were varied at three levels. The results indicate that less variability in retention time and tailing factor were observed. (Table No. 24, 25).

Discussion :

The analysis of tablet formulation was done and the results obtained within the limits. The results obtained for validation study were within the limit specified by the ICH guidelines and hence the method was found to be linear, precise. The results of recovery study were within ICH limits, thus indicating the accuracy.

SUMMARY & CONCLUSION

1) The method provides selective quantification of Sofosbuvir and Daclatasvir. This developed RP-HPLC method for estimation sofosbuvir and Daclatasvir is accurate, precise and robust.

2) The method has been found to be better than previously reported method, because of its less retention time, gradient mode and use of economical readily available mobile phase, readily available column, UV detection and better resolution of peaks.

3) The run time is relatively short, which will enable rapid quantification many samples in routine and quality controlled analysis of various formulations containing sofosbuvir and Daclatasvir. All these factors make this method suitable for quantification of sofosbuvir and Daclatasvir pharmaceutical dosage forms without any interference.

4) The method was completely validated showing satisfactory data for all the method validation parameters tested. Hence this method can be introduced into routine use for determination of sofosbuvir and Daclatasvir.

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