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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF SOFOSBUVIR AND LEDIPASVIR: A PHARMACEUTICAL APPLICATION STUDY

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ABSTRACT

The objective of the present work was to develop a simple, precise, accurate, reproducible, and specific reversed-phase high performance liquid chromatographic method for the simultaneous determination of Sofosbuvir and Ledipasvir in pharmaceutical dosage form. The HPLC system (Shimadzu, LC-2030) consisted of Hypersil ODS - C18 (250 mm, 4.6 mm, 5 μm- Thermo Scientific) was used for the isocratic elution using mobile phase composed of Sodium Phosphate buffer (pH 3.5) and Acetonitrile in the ratio of 40:60% (v/v). Both Sofosbuvir and Ledipasvir were detected at 260 nm with a flow rate of 1 ml/min. The mean retention time of Sofosbuvir was 3.094, whereas Ledipasvir was 6.22 minutes. A linear relationship was found in the concentration ranged from 0.54 to 162 µg/mL for Sofosbuvir (r^2 = 0.9993) and 0.14 to 42 µg/mL for Ledipasvir (r^2 = 0.9994). The RSD values for recovery test were found 0.99% for Sofosbuvir and 0.22% for Ledipasvir. The lower limit of detection (LOD) were 0.054 mcg/mL and 0.012 mcg/mL, and the limit of quantification (LOQ) were 0.135 mcg/mL and 0.030 mcg/mL for Sofosbuvir and Ledipasvir, respectively. The method was successfully applied to the assay and *in-vitro* dissolution studies of Sofosbuvir and Ledipasvir in tablet dosage form and all the results were found within the acceptance limits.

***Keywords*:** Sofosbuvir, Ledipasvir, RP-HPLC, method validation, pharmaceutical application

INTRODUCTION

Sofosbuvir and Ledipasvir are very effective drugs against hepatitis C virus (HCV). Sofosbuvir is a nucleotide analogue polymerase inhibitor of non-structural protein (NS-5B), efficacious against every genotypes of HCV, with and without pegylated interferon **[1, 2]**. Similarly, Ledipasvir is also an inhibitor of non-structural protein 5A (NS-5A), effective against HCV genotypes 1a and 1b. It is used as a combination therapy with Sofosbuvir to treat chronic Hepatitis C **[3]**. The chemical name of Sofosbuvir is isopropyl (2S)-2-{[(S)-{[5-(2, 4-dioxo-3, 4-dihydro-1(2H)-pyrimidinyl)-4-fluoro-3-hydroxy-4-methyltetrahydro-2-furanyl]methoxy} phenoxy) phosphoryl] amino} propanoate, whereas, chemical name of Ledipasvir is (2S)-1-[(6S)-6-[5-(9,9-difluoro-7-{2-

[(1R,3S,4S)-2-[(2S)-2-{[hydroxy(methoxy)methylidene]amino}-3-methylbutanoyl]-2-azabicyclo [2.2.1]heptan-3-yl] -1H-1,3-benzodiazol-6-yl} -9H-fluoren-2-yl) -1H-imidazole-2-yl] -5-azaspiro[2.4] heptan-5-yl] -2-{[hydroxyl (methoxy) methylidene] amino}-3-methylbutan-1-one **[4]**, as shown in figure 1 and 2, respectively. Currently, number of life-threatening diseases are arising which are more harmful and dangerous to the human being like hepatitis, AIDS and different types of cancer etc. Hepatitis is a global health problem which has currently infected more than 0.170 billion people all over the world, causing more than 0.35 million death per year **[5-8]**. Hepatitis C is an infectious liver disease caused by the hepatitis C virus (HCV), a single-stranded RNA virus. Approximately 3.2 million people have chronic HCV infection in the United States and among them, 5 – 20% eventually develop to liver cirrhosis whereas, 1 – 5% die from cirrhosis or hepatocellular carcinoma. Due to hepatitis C-related liver diseases, approximately 0.7 million people die per year **[8, 9]**. Validation is a process of collection of documentary evidence that any of the procedure, process, method, or activity being adapted is capable of producing consistent and satisfactory result in terms of measurements or in terms of product quality. To demonstrate that a pharmaceutical product manufactured with any process in any pharmaceutical company, it is required to validate many procedures, processes, methods activities associated with pharmaceutical manufacturing including machinery, skills, testing procedures and methods **[10, 11]**. The literature survey showed that several methods have been reported for the estimation of Sofosbuvir and Ledipasvir in solid dosage form **[12-14]**. In the present study, a simple, economical, precise, accurate RP-HPLC method has been developed and validated for the simultaneous quantitative determination of Sofosbuvir and Ledipasvir from the tablet dosage form.

MATERIALS AND METHODS

Chemicals and reagents

Sofosbuvir and Ledipasvir (Reference standard) were kindly gifted by PharmEvo Ltd. HPLC grade phosphoric acid, polysorbate 80, sodium dihydrogen phosphate and potassium dihydrogen phosphate were purchased from Merck KGaA, Darmstadt, Germany. Butylated hydroxytoluene (BHT) was procured from VWR USA. Highly purified distilled water collected from plant of PharmEvo private limited. Brand of Sofosbuvir and Ledipasvir tablets were purchased from well reputed retail pharmacy, Karachi. All other chemicals and reagents used in the current study were of HPLC grade quality.

Instrumentation

High Performance Liquid Chromatographic system (Shimadzu LC-2030 Prominence-I Series), consisted of LC-20AT VP pump, and Hypersil ODS C18-Column (250mm x 4.6mm, 5µm -Thermo Scientific), was used in the study. Moreover, dissolution apparatus, (Pharmatest Germany), analytical Balance (Mettler Toledo Germany), Disintegration apparatus (Pharmatest Germany), PH Meter (WTW Ino Lab -

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Germany), Sonicator (Elma Germany), Filtration assembly (Sartorius, Gorringen, Germany), Vacuum pump (China) and Vernier caliper (China) were also used in the study.

Chromatographic conditions

For the separation of Sofosbuvir and Ledipasvir, an isocratic HPLC system equipped with UV detector (Schimadzu), LC-20AT VP pump and Hypersil ODS C18 (250 mm × 4.6 mm, 5µm) column was used, and the chromatograph were recorded using LC Solution software. The sample was detected at 260 nm wavelength with a flow rate of 1.0ml/min. The volume of injection was 10 µL with a run time of 8 minutes. The retention time of Sofosbuvir and Ledipasvir were 3.103 min and 6.241 min., respectively. Figure 3 shows the chromatogram of Sofosbuvir and Ledipasvir.

Preparation of buffer solution and mobile phase

Accurately 6.8 g of sodium dihydrogen phosphate was weighed, transferred to 1000 mL volumetric flask, and sonicated to dissolve in distilled water. This phosphate buffer was adjusted at pH 3.5 with phosphoric acid. The mobile phase was prepared using phosphate buffer and acetonitrile at a ratio of 60:40 (v/v), respectively. The mobile phase was filtered through membrane filter (0.45µm) with the help of filtration assembly and sonicated before use in order to degas the mobile phase.

Preparation of stock and working standard solutions

For standard stock solution, accurately 108 mg of Sofosbuvir and 24 mg of Ledipasvir were weighed and completely dissolved in HPLC grade water into 100 mL volumetric flask separately. Standard working solutions of Sofosbuvir and Ledipasvir were prepared by transferring 5 mL of 1st stock µstandard (Sofosbuvir) and 5 mL of 2nd stock standard solution (Ledipasvir) into a separate 100 mL volumetric flask, diluted and mixed with mobile phase to obtain 54 µg/mL and 12 µg/mL, sequentially. Before injecting into the HPLC system (10 µL), the working standard solutions were then filtered through 0.45µ membrane filter.

Preparation of samples from pharmaceutical marketed product

Commercially available tablet brand, each containing 400 mg of Sofosbuvir and 90 mg of Ledipasvir was used for preparation of samples. Twenty tablets were accurately weighed and triturated to obtain a homogeneous mixture. Accurately weighed 250 mg of powder (equivalent to 108 mg of Sofosbuvir & 24 mg of Ledipasvir) was transferred into 100 ml volumetric flask containing 25 mL of diluent to dissolve & sonicated for 25 minutes. Again, mixed magnetically for 30 minutes and made-up volume with diluent. This solution was further diluted by pipetting 5 mL into 100 mL volumetric flask and volume made up using mobile phase to obtain a concentration of 54 µg/mL and 12 µg/mL, accordingly, followed by filtration through 0.45µ before injecting into system (10 µL).

Selection of wavelength

Spectrophotometer was used for scanning of the Sofosbuvir and Ledipasvir standards in the UV region (200-400 nm) to obtain the

wavelength at which maximum absorbance achieved.

The final detection of the drug was then carried out at 260 nm wavelength. After successful scanning, different samples of tablets were also run on HPLC at different wavelength and 260 nm wavelength was found satisfactory for both Sofosbuvir and Ledipasvir. The peak of Sofosbuvir and Ledipasvir was detected at 3.103 min and 6.241 minutes, respectively.

Stability of sample solutions

It is an important requirement to analyze the stability of samples quantitively. The stability of samples solution was performed by keeping the samples at room temperature for 12h. Similarly, evaluation was also conducted at -15 to -20°C for 7 days **[15]**.

Method validation

The developed method of analysis was validated according to International Council for Harmonization (ICH) guideline for the parameters such as system suitability, specificity, linearity, accuracy and precision, limit of detection (LOD) and limit of quantification (LOQ).

System suitability

Initially, the HPLC system was stabilized for 40 minutes. The system suitability parameters were assessed by repetitively injecting six replicates of drug solution at a concentration of 54 µg/mL (Sofosbuvir) and 12 µg/mL (Ledipasvir), to check the reproducibility of the system. The system suitability of the developed method was check by recording different parameters like, peak area, % RSD, retention time and tailing factor.

Specificity

The effect of excipients and other additives usually present in the tablet formulation of Sofosbuvir and Ledipasvir was evaluated. In order to determine the specificity of the analytical method, the most commonly used excipients such as lactose anhydrous, microcrystalline cellulose and magnesium stearate have been incorporated in the solution containing Sofosbuvir (54 µg/mL) and Ledipasvir (24 μg/mL) was injected (triplicate) and tested. The representative chromatogram showed no additional peak other than drug was observed. The mean % recovery for Sofosbuvir (101.59 %) and Ledipasvir (99.02%), also verified that this method could be employed to estimate the Sofosbuvir and Ledipasvir in pharmaceutical dosage forms **[15]**.

Linearity

Approximately, fourteen concentrations (0.5% to 150%) i.e. 0.543, 1.086, 5.423, 10.865, 21.730, 54.324, 65.189, 86.918, 97.783, 108.648, 119.513, 130.378, 141.544, 177.056 µg/mL were injected, in order to assess the linearity **[16]**. Method of least square analysis was applied for the determination of coefficient of correlation (R^2) value. A calibration curve of Sofosbuvir and Ledipasvir were plotted between concentration versus mean area response and statistical analysis of the calibration curve are shown in figure 4 and 5, accordingly.

Accuracy (Recovery studies) and precision

The accuracy of an analytical procedure expresses the degree of closeness of agreement between the values which is accepted either as a conventional true value

or an accepted reference value and the value found **[17, 18]**. Accuracy of the method was determined by recovery study, in which three different known concentration levels (80%, 100% and 120%) of Sofosbuvir and Ledipasvir solutions were added to the samples containing known contents and the percentage of recovery was calculated by comparing the determined amount of these standards with the amount added to the samples. Table 2 and 3 shows the mean percentage recovery of Sofosbuvir and Ledipasvir at each level, observed in the range of 100.427 – 102.221% and 98.795 – 99.246%, respectively **[19]**. The method was also validated for intra- and inter-day precisions for both drugs. Precision of the anticipated method was evaluated by repeatability (inter-day precision) and intermediate precision (intra-day precision) at six different weight levels ranges from 244.7 – 253.6 mg. Intraday precision of Sofosbuvir and Ledipasvir were carried out on the same day, whereas intraday precision was performed on the different days. The percentage relative standard deviation (RSD) of both intra as well as intraday were calculated and results of drugs are given in table 5 and 6, respectively. The calculated RSD should be less than 2%.

Limit of detection (LOD) and Limit of quantitation (LOQ)

Limit of detection is the lowest concentration in a sample that can be detected, but not quantified under the same experimental condition, whereas limit of quantification (LOQ) is the lowest concentration of drug in the sample that can be quantified with acceptable accuracy and precision. LOD and LOQ were determined by analyzing seven samples of different lower concentrations such as 54.32, 21.73, 10.87, 5.42, 1.09, 0.54, 0.27 µg/mL (Sofosbuvir) and 11.86, 5.31, 2.66, 1.33, 0.33, 0.12, 0.06 µg/mL (Ledipasvir) as shown in table 6 and 7.

Estimation of Sofosbuvir and Ledipasvir in the commercial brand using this method

The potency of the Sofosbuvir and Ledipasvir present in the marketed brand was also determined by using the current developed and validated method and the result is given table 8.

RESULTS

Linearity, system suitability and precision

The method revealed good linearity with correlation (R^2) values of 0.9993 (Sofosbuvir) and 0.9994 (Ledipasvir) as shown in figure 4 and 5. System was evaluated on each day of method validation by injecting 14 runs of standard samples. System suitability parameters were evaluated like, %RSD (Relative Standard Deviation), retention time, peak area and tailing factor **[16, 20]**, which were found within the range (table 1). The method was also validated for intra-day and inter-day accuracy and precision for both drugs. The intraday accuracy of Sofosbuvir was 102.33% and Ledipasvir was 99.28%. Whereas intraday accuracy of Sofosbuvir was 102.39%, while, Ledipasvir was 99.18%, as shown in Table 4 and 5.

Analytical recovery study

Percentage recovery tests of both drugs were conducted by adding known amounts of standard solutions to sample followed by

analysis using proposed method. Three runs were conducted for each concentration and then peak area was calculated and results are shown in table 2 and 3.

Limit of detection (LOD), limit of quantification (LOQ)

LOD for Sofosbuvir and Ledipasvir were found as 0.540 and 0.120 µg/mL, whereas LOQ of Sofosbuvir and Ledipasvir were observed as 1.09 and 0.330 µg/mL, accordingly and results are given in Table 6 and 7.

Assay of commercial brand

The assay of marketed tablet brand containing Sofosbuvir and Ledipasvir, was also conducted by using the current developed and validated method and the potency was found 102.64 and 99.25%, respectively, as given in table 8.

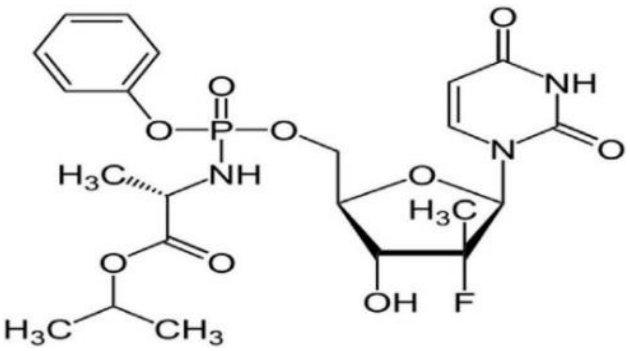

Figure 1: Structure of Sofosbuvir

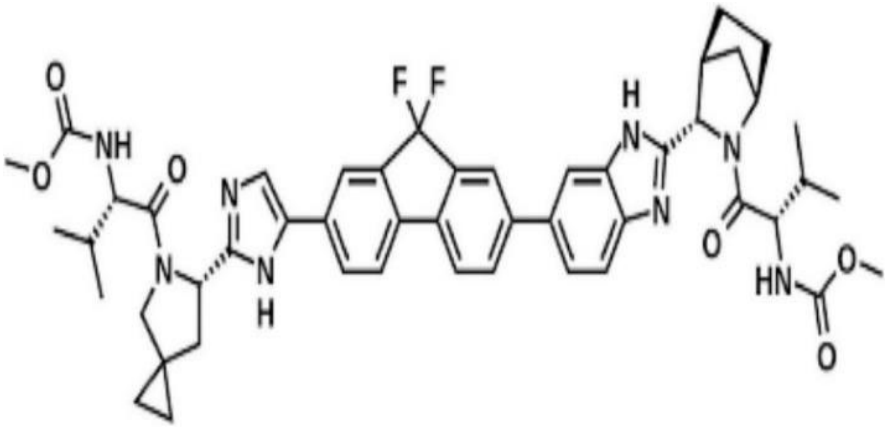

Figure 2: Structure of Ledipasvir

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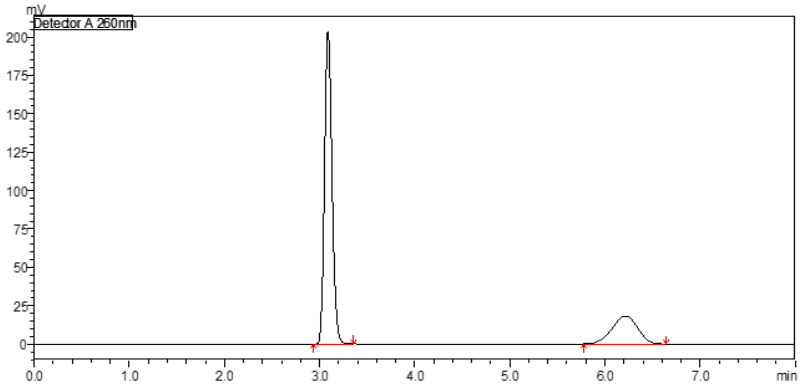

Figure 3: Chromatograms for Sofosbuvir and Ledipasvir

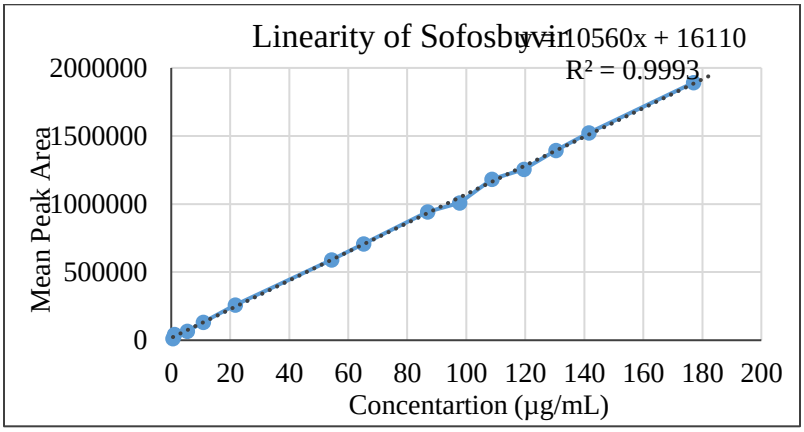

Figure 4: Linearity graph of Sofosbuvir

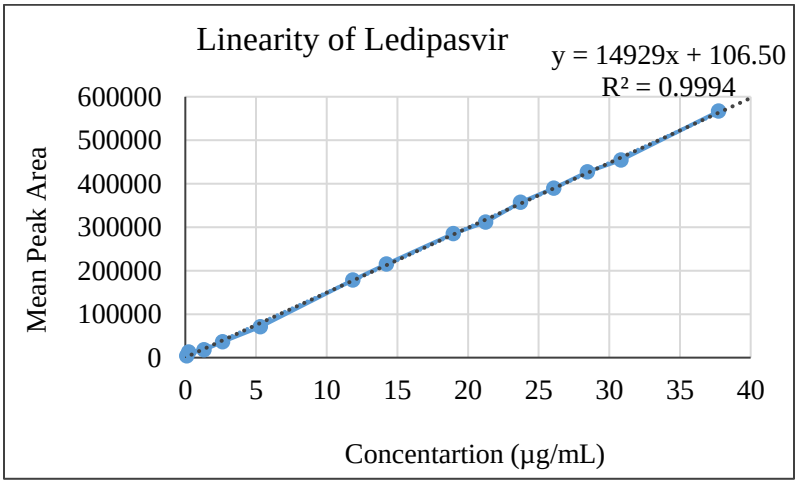

Figure 5: Linearity graph of Ledipasvir

DISCUSSION

Sofosbuvir is one of the best drugs to treat the hepatitis C infection. Many scientists across the globe doing research on these drugs and solid dosage forms (tablet) has prepared for the treatment of infection. Sofosbuvir in combination with Ledipasvir is very effective for the treatment of the Hepatitis C Infection. It is available in a fixed dose of tablet form containing 400mg Sofosbuvir and 90mg of Ledipasvir, which was developed by Gilead Sciences, Inc in the name of brand "Harvoni" **[21]** and it was approved by FDA in October, 2014. Different pharmaceuticals industries producing medicines having the same generic like Sofosbuvir and Ledipasvir for the management of hepatitis C infection. To manufacture a good quality product, the drug should be tested as per international standards or guidelines. The quality personals and researchers continuously trying to develop and validate an analytical method which should be accurate, easy, and reproducible.

In this study, an effort has been made to develop a simple, isocratic, accurate and sensitive RP-HPLC method for the simultaneous determination of Sofosbuvir and Ledipasvir in pharmaceutical products. Hypersil ODS C18 (250mm × 4.6mm, 5µm) column was used for separation, which provided the best peak shapes and efficiencies. The method was validated as per International Council for Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH, 2002). All the parameters such as specificity, linearity, accuracy, precision, and percent recovery were found within the acceptable limits. The best resolution, separation and no interference from the mobile phase appeared at 260 nm. The injection volume and run time were 10µL and 8 min, respectively. The mean retention time of Sofosbuvir was 3.103 min., whereas Ledipasvir was 6.241 minutes.

Linear regression by the least square method was applied for the assessment of coefficient of correlation for linearity, which were 0.9993 (Sofosbuvir) and 0.9994 (Ledipasvir), indicating excellent linearity (figure 4 and 5). The evaluated system suitability parameters were, %RSD, retention time, peak area and tailing factor, which were found within the acceptance range (see in table 1). Zaman *et al.*, also reported similar result **[13]**. Previous study indicated similar in concentration range (40.0 to 500.0 µg/ml) for Sofosbuvir and (30.0 to 300.0 µg/ml) for Ledipasvir for assay content and dissolution rate and r^2 values were 0.9996 and 0.9993 for Sofosbuvir and Ledipasvir **[22]**. Another study of Sofosbuvir, showed a linear relationship with r^2 values of 0.9990 **[23]**. The precision is one the most important step of the method validation. Precision of the anticipated method was determined by inter-day precision and intra-day precision, which was expressed as relative standard deviation (RSD) as given in table 4 and 5. Similar types of findings was also reported in a previous study **[24]**.

The average %age recovery for each level was calculated as per association of official analytical chemists international **[18, 20, 25]**. The percentage recovery for accuracy of

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Sofosbuvir was 101.59%, and Ledipasvir was 99.02%. It was concluded that the excipients used in the formulation did not interfere with drug present in tablets and selected medium did not absorb the drug to any extent. The obtained percentage recovery indicated that the method has a high degree of accuracy for simultaneous estimation of Sofosbuvir and Ledipasvir (Table 2 and 3). LOD for Sofosbuvir and Ledipasvir were found as 0.540 and 0.120 μg/mL, whereas LOQ of Sofosbuvir and Ledipasvir were observed as 1.09 and 0.330 μg/mL, accordingly, (table 6 and 7), indicating that the method could be very useful for any country following relevant quality standards (table 5). In the previous study different buffers were used like ammonium dihydrogen phosphate and pH also changed at basic side indicating the method is more precise **[13]**. By using the current developed and validated method, assay results of marketed tablet brand of Sofosbuvir and Ledipasvir were found 102.64 and 99.25%, respectively, as shown in Table 8.

Table 1: System suitability peak area of Sofosbuvir and Ledipasvir

n #	Peak area of Sofosbuvir	Peak area of Ledipasvir
Run 1	1116578	343599
Run 2	1117227	344192
Run 3	1117805	343832
Run 4	1117499	344128
Run 5	1117360	343596
Run 6	1117160	343640
Mean Peak area	1117270	343831
Standard Deviation (SD)	409.772	269.837
% RSD	0.037	0.078
Mean Retention time (min.)	3.103	6.241
Tailing Factor	1.169	0.938

Table 2: Percentage recovery for accuracy of Sofosbuvir

No of sample	Amount/ conc. of drug	Peak Area (Run A)	Peak Area (Run B)	Peak Area (Run C)	Mean Peak Area	% drug found	% recovered
1	80 %	949636	949301	949685	949540.667	81.78	102.221
2	100 %	1186711	1186780	1186594	1186594	102.12	102.117
3	120 %	1401332	1404079	1399407	1401606	120.51	100.427
Mean					101.59 %		
Standard Deviation (SD)					1.01		
Relative standard deviation (RSD) (Limit: NMT 2.0%)					0.99 %		

Table 3: Percentage recovery for accuracy of Ledipasvir

No of sample	Amount/ conc. of drug	Peak Area (Run A)	Peak Area (Run B)	Peak Area (Run C)	Mean Peak Area	% drug found	% recovered
1	80 %	286685	286831	286841	286845.67	79.40	99.246
2	100 %	357886	358248	358066	358066.67	99.03	99.029
3	120 %	428895	429296	428865	429018.67	118.55	98.795
Mean					99.02 %		
Standard Deviation (SD)					0.23		
Relative standard deviation (RSD) (Limit: NMT 2.0%)					0.29 %		

Table 4: Intraday precision characteristics of Sofosbuvir and Ledipasvir

Injection volume (µL)	Theoretical Percentage (%)	Weight of Sample (mg)	Sofosbuvir		Ledipasvir	
			Mean Peak area	Percentage found (%)	Mean Peak area	Percentage found (%)
10	100	247.1	1127097	102.37	345725	102.37
10	100	247.2	1130867	102.68	348084	102.68
10	100	247.6	1127305	102.19	347236	102.19
10	100	247.3	1122652	101.89	346043	101.89
10	100	253.3	1157511	102.56	357200	102.56
10	100	253.6	1156128	102.32	356220	102.32
Mean			102.33 %		99.28 %	
Standard Deviation (SD)			0.28		0.32	
Relative standard deviation (RSD) (Limit: NMT 2.0%)			0.27 %		0.32 %	

Table 5: Interday precision characteristics of Sofosbuvir and Ledipasvir

Injection volume (µL)	Theoretical Percentage (%)	Weight of Sample (mg)	Sofosbuvir		Ledipasvir	
			Mean Peak area	Percentage found (%)	Mean Peak area	Percentage found (%)
10	100	245.0	1132554	101.83	347937	98.34
10	100	245.2	1140631	102.47	351691	99.32
10	100	245.3	1131789	101.63	348650	98.43
10	100	244.7	1133289	102.02	349539	98.92
10	100	248.0	1164373	103.42	358904	100.22
10	100	248.9	1163463	102.97	358811	99.83
Mean			102.39 %		99.18 %	
Standard Deviation (SD)			0.70		0.76	
Relative standard deviation (RSD) (Limit: NMT 2.0%)			0.68 %		0.76 %	

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Table 6: Limits of detection and limit of quantification for Sofosbuvir

Concentration (µg/ml)	Back Calculated Concentration (µg/ml)					Mean	SD	% Assay	Peak area
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5				
54.32	54.301	54.324	54.33	54.228	54.198	54.2762	0.060	99.91	590583
21.73	22.895	22.119	22.611	22.66	22.181	22.542	0.332	103.74	259424
10.87	10.754	10.759	10.752	10.754	10.758	10.755	0.003	98.99	131410
5.42	5.345	5.441	5.135	5.241	5.325	5.307	0.115	97.86	66330
1.09	1.111	1.105	1.099	1.124	1.101	1.105	0.010	101.75	18112
0.54	0.009	0.0086	0.0098	0.0039	0.0096	0.009	0.002	1.68	9106
0.27	-	-	-	-	-	-	-	-	Not detected

Table 7: Limits of detection and limit of quantification for Ledipasvir

Concentration (µg/ml)	Back Calculated Concentration (µg/ml)					Mean	SD	% Assay	Peak area
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5				
11.86	12.007	11.989	12.012	11.98	12.221	12.003	0.101	101.22	178662
5.31	5.181	5.205	5.298	5.248	5.278	5.228	0.049	98.42	76324
2.66	2.602	2.598	2.756	2.754	2.699	2.652	0.078	99.85	37660
1.33	1.283	1.301	1.401	1.411	1.315	1.328	0.059	100.03	17901
0.33	0.316	0.333	0.301	0.341	0.322	0.317	0.015	96.84	2202
0.12	0.079	0.101	0.098	0.111	0.087	0.093	0.012	77.87	1010
0.06	-	-	-	-	-	-	-	-	Not detected

Table 8: Determination of drug content present in commercial brand of Sofosbuvir and Ledipasvir by this proposed method.

Analyte	Measured amount Mean ± SD	Claimed amount (mg)	% potency *
Sofosbuvir	410.56 ± 0.49	400	102.64 %
Ledipasvir	89.325 ± 0.46	90	99.25 %
*** Potency = Measured amount × 100/Claimed amount**			

CONCLUSION

It was concluded that during the present study, a simple, accurate, rapid, precise, reproducible and inexpensive RP-HPLC method was developed with excellent correlation co-efficient for the simultaneous estimation of Sofosbuvir and Ledipasvir in pharmaceutical products. The developed method was validated according to ICH guidelines for validation of analytical procedure. Moreover, the mobile phase is cheap one and easily available. The shorter retention time made the method easier, less time consuming for analysis and applicable for pharmaceutical industry, especially in quality control department.

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