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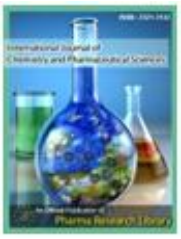

Research Article Open Access

RP-HPLC Method Development and Validation for Simultaneous Estimation of Ledipasvir and Sofosbuvir in Bulk and Tablets

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A B S T R A C T

The present paper describes a new, simple, precise, and accurate RP-HPLC method for simultaneous estimation of ledipasvir and sofosbuvir in bulk and formulation. Separation was performed on Kromacil-100 HD,C18 column (15cm x 4.6mm i.e., 5 µm) at ambient temperature. The mobile phase consisted of ammonium acetate buffer (pH 6.0), acetonitrile and methanol in the ratio of 30:40:30 (v/v) at a flow rate of 1.0 ml/min using isocratic pump system. Detection was carried out at wavelength 285 nm. The retention times of ledipasvir and sofosbuvir were 3.06 min and 7.45 min, respectively. The linearity was established over the concentration ranges of 50-175µg/ml with correlation coefficients of 0.998 and 0.999 for ledipasvir and sofosbuvir, respectively. The mean recoveries were found to be in the ranges of ledipasvir was 98.7-100.1% and sofosbuvir was 98.8-100.1% respectively. The proposed method has been validated as per ICH guidelines and successfully applied to the estimation of ledipasvir and sofosbuvir in their combined tablet dosage form.

Keywords: RP-HPLC, Method development, Validation, Simultaneous estimation, Robustness.

A R T I C L E I N F O

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1. Introduction

Ledipasvir and sofosbuvir is a combination of hepatitis C virus (HCV) antiviral agents 1.It reduces the amount of HCV in the body by preventing the spread of the HCV within the body. Ledipasvir is a Hepatitis C virus NS5A

inhibitor with molecular formula of C71H83F3N11O25P chemically described as Methyl [(2S)-1-{(1R,3S,4S)-3-[5-(9,9-difluoro-7-{2-[(6S)-5-{(2S)-2-[(methoxycarbonyl) amino]-3-methylbutanoyl}-5-azaspiro[2.4]hept-6-yl]-1H imidazol-4-yl}-9H-fluoren-2-yl)-1H-benzimidazol-2-yl]-2-azabi cyclo[2.2.1]hept-2-yl}-3-methyl-1-oxo-2-butanyl] carbamate2. The mechanism of action of ledipasvir is as a P-Glycoprotein inhibitor, and breast cancer resistance protein inhibitor. Ledipasvir is an orally available inhibitor of the hepatitis C virus (HCV) non-structural protein 5A (NS5A) replication complex, with potential activity against HCV. Upon oral administration and after intracellular uptake, ledipasvir binds to and blocks the activity of the NS5A protein3. This results in the disruption of the viral RNA replication complex, blockage of HCV RNA production, and inhibition of viral replication. NS5A, a zinc-binding and proline-rich hydrophilic phosphoprotein 4, plays a crucial role in HCV RNA replication. HCV is a small, enveloped, single-stranded RNA virus belonging to the flaviviridae family; HCV infection is associated with the development of hepatocellular carcinoma (HCC). Sofosbuvir with a molecular formula of C22H29FN3O9P, chemically described as (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.

Sofosbuvir and other nucleotide inhibitors of the HCV RNA polymerase exhibit a very high barrier to resistance development. Sofosbuvir 5 is potent in inhibiting the HCV NS5B RNA-dependent RNA polymerase, which is responsible for viral replication. Being a nucleotide prodrug it undergoes intracellular metabolism to produce GS-461203, active uridine analog triphosphate 6 and exhibits action by incorporation into HCV RNA by chain termination facilitated by NS5B polymerase. This is an important advantage relative to HCV drugs that target other viral enzymes such as the protease 7 , for which rapid resistance development has proved to be an important cause of therapeutic failure. Sofosbuvir has become available as fixed dose drug combination product with ledipasvir used for the treatment of chronic Hepatitis C. Recently few methods are reported for individual and simultaneous determination of these drugs. Sofosbuvir in pure form, in bulk and tablet dosage form was determined by RP-HPLC.

Finally, sofosbuvir was used as an internal standard (IS) in an UPLC-MS/MS method for the determination of daclatasvir 8(DAC) in human plasma. While for ledipasvor, only two methods have been published for its individual determination in bulk drug form by simple UV spectrophotometry and by RP-HPLC. Both sofosbuvir and ledipasvir in human plasma were determined by UPLC-MS/MS 9 method and besides some antiviral agents. Ledipasvir, sofosbuvir and its metabolite in rat plasma were also, determined by UPLC-MS/MS .So an attempt was done to develop simple, precise ,rapid accurate RP-HPLC method for the simultaneous determination of both sofosbuvir and ledipasvir together in pure and tablet dosage forms. Validation of the developed methods was performed according to ICH guidelines.

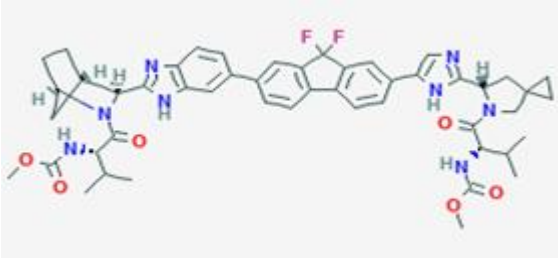

Figure 1: Structure of ledipasvir

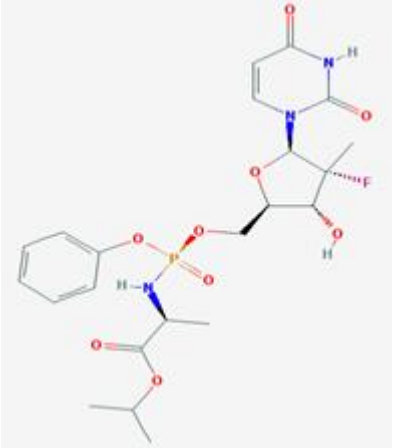

Figure 2: Structure of sofosbuvir

2. Materials and Methods

Apparatus and chromatographic condition:

The chromatographic separation performed with Schimadzu LC system equipped with LC-20 AT pump and SPD-20 AV detector and rheodyne 7725 injector with fixed loop of 20 µl. Chromatographic separation was carried out on a Kromacil-100 HD,C18 column (15cm x 4.6mm i.e., 5 µm) with mobile phase composed of ammonium acetate buffer (pH 6.0)acetonitrile and methanol in the ratio of 30:40:30 (v/v) at a flow rate of 1.0 ml/min. The mobile phase was prepared, filtered, sonicated before use and the detection wavelength was set at 285 nm. In addition pH meter, an electronic balance and sonicator were used.

Materials, chemicals and reagents

Ledipasvir and Sofosbuvir standard was provided by Hetero Pharma Ltd as gift samples. Marketed formulation containing ledipasvir 50 mg and sofosbuvir 5 mg were obtained from local market. Analytical grade ammonium acetate was purchased from S.S. fine chemicals, Hyderabad. HPLC grade acetonitrile and water were obtained from Merck, Mumbai

Wavelength detection:

In simultaneous estimation[10] of two drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate both drugs accurately. The wavelength of maximum absorption (λ_{max}) of the drug, 10 µg/ml solution of the drugs in methanol were scanned using UV-Visible spectrophotometer within the wavelength

region of 200–400 nm against methanol as blank. The isobestic point[11] was found to be 285 nm for the combination.

Method Development

Preparation of standard stock solution of ledipasvir

Ledipasvir 10 mg was weighed and transferred in to 100ml volumetric flask and dissolved in water and then make up to the mark with water and prepare 40 µg /ml of solution by diluting 4ml to 10ml with water.

Preparation of standard stock solution of sofosbuvir

Sofosbuvir 10 mg was weighed and transferred in to 100ml volumetric flask and dissolved in water and then dilute up to the mark with water and prepare 30 µg /ml of solution by diluting 3ml to 10ml with water.

Preparation of mixed standard solution

Accurately weighed and transferred 10mg of ledipasvir and sofosbuvir in to 100 ml volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of ledipasvir and of sofosbuvir is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Preparation of sample solution

Ten tablets (each tablet contains sofosbuvir -5 mg, ledipasvir -50 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of sofosbuvir and ledipasvir (µg/ml) were prepared by dissolving weight equivalent to 10 mg of sofosbuvir and ledipasvir and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and sonicated for 5 min and dilute to 10ml with mobile phase. Further dilutions are prepared in five replicates of 10µg/ml of sofosbuvir and ledipasvir was made by adding 1 ml of stock solution to 10 ml of mobile phase.

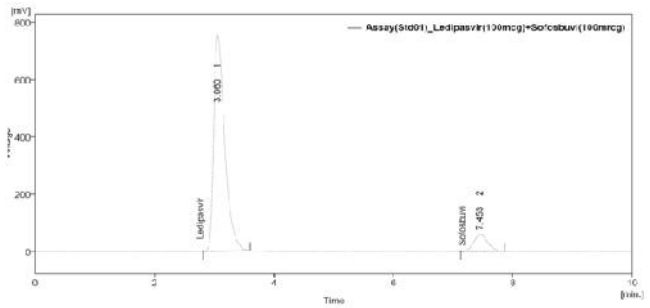

Figure 3: Chromatogram of assay of standard preparation

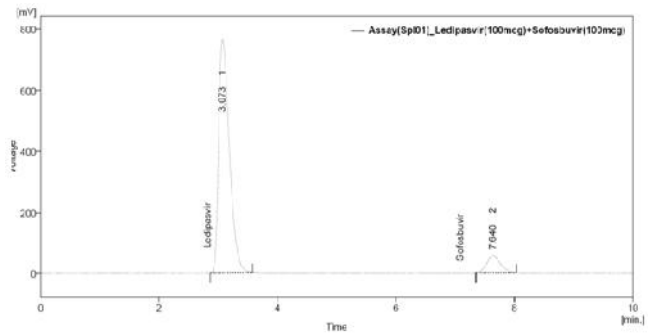

Figure 4: Chromatogram of assay of sample preparation

Calculation

The amount of sofosbuvir and ledipasvir 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: Peak area due to sample preparation
WS: Weight of standard in mg
WT: Weight of sample in mg
DT: Dilution of assay preparation
AW: Average weight of sample
LC: Label claim
P: Potency of pure drug

3. Results and Discussion

Method Validation

Linearity:

Five linear concentrations of ledipasvir (50-175µg/ml) and sofosbuvir (50-175µg/ml µg/ml) are prepared and injected. Regression equation of the ledipasvir and sofosbuvir found to be y = 29.69x + 6646 and y=4.214x + 566.0 with coefficient of determination of 0.998 and 0.999. Linearity solutions are prepared such that 5 ml, 7.5ml, 10 ml, 15ml, 17.5ml, from the Stock solutions of ledipasvir and sofosbuvir are taken in to five different volumetric flasks and diluted to 10ml with diluents to get 50, 75, 100, 150, 175 µg/ml of ledipasvir and sofosbuvir.

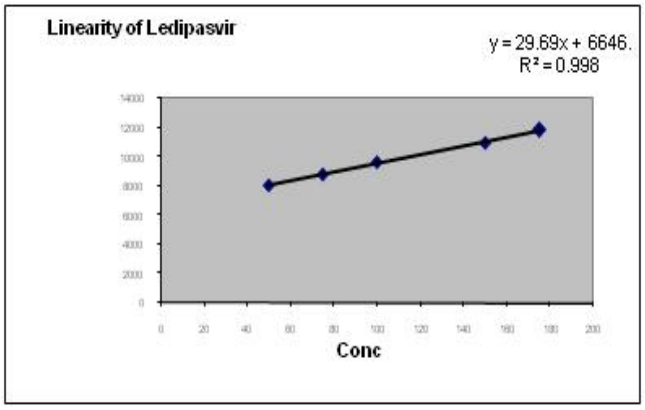

Figure 5: Calibration curve of ledipasvir

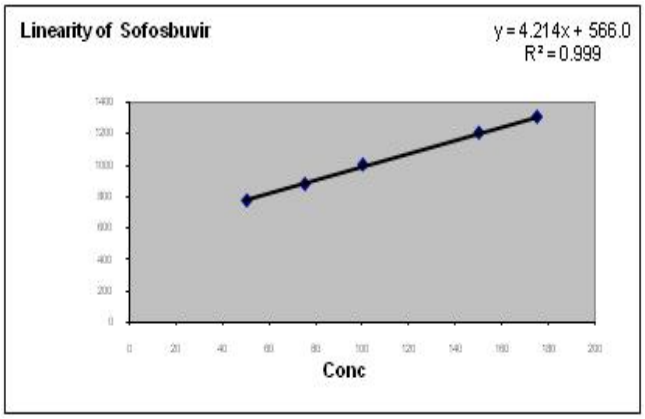

Figure 6: Calibration curve of sofosbuvir

The coefficient of determination for linear curve obtained between concentration vs. area for standard preparations of ledipasvir and sofosbuvir is 0.998 and 0.999. The relationship between the concentration of ledipasvir and sofosbuvir and area is linear in the range examined since all points lie in a straight line and the coefficient of determination is well within limits.

Limit of detection and Limit of quantitation study[12]:

LOD is the lowest amount of the drug content which can be detected by the proposed method while LOQ is the lowest amount which can be quantified by the method. The guideline suggests minimum signal to noise ratio (S/N) more than 3.3 for LOD and more than 10 for LOQ. On the basis of linearity data theoretically it can be also calculated by the given formula.

LOD=3.3 σ/S
LOQ=10 σ/S
Where σ = residual standard deviation of regression line and S=slope of regression line

LOD for ledipasvir and sofosbuvir were found to be 0.30 µg/ml and 0.45 µg/ml respectively.
LOQ for ledipasvir and sofosbuvir were found to be 0.95 µg/ml and 1.3 µg/ml respectively.

System Precision

Precision[13] study was established by evaluating method precision and system precision study. Method precision of the analytical method was determined by analyzing six sets of the sample preparation. Assay of all six replicate sample preparation was determined and mean % assay value, standard deviation and% RSD for the same was calculated. System precision of the analytical method was carried out to ensure that the analytical system was working properly. Standard solution was injected six times in to system and chromatograms were recorded.

Preparation of standard solution [14]:

Accurately weighed and transferred 10mg of ledipasvir and sofosbuvir in to 100 ml volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of ledipasvir and of sofosbuvir is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Table 1: System precision studies of ledipasvir

Ledipasvir		
S.No.	**Rt**	**Area**
1	3.073	9798.904
2	3.073	9544.612
3	3.073	9633.752
4	3.070	9588.314
5	3.073	9695.748
6	3.060	9714.063
Avg	3.0703	9662.566
Stdev	0.0052	92.301
%RSD	**0.17**	**0.96**

Table 2: System precision studies of sofosbuvir

Sofosbuvir		
S.No.	**Rt**	**Area**
1	7.640	954.959
2	7.497	970.903
3	7.460	945.723
4	7.443	964.109
5	7.640	941.663
6	7.453	935.153
Avg	7.522	952.085
Stdev	0.093	13.731
%RSD	**1.24**	**1.44**

The area response and assay values are consistent as evidenced by the values of relative standard deviation. Hence it can be concluded that the system parameter and method parameters meets the requirement of method validation.

Accuracy:

Accuracy of the method was determined by recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery [15] studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre analyzed sample solution at three different levels 50%, 100%, 150%.

Robustness Study

Robustness of the method was evaluated by assaying test solution under slight but deliberate changes in analytical conditions, such as change in flow rate, change in mobile phase composition, change in column and change in wave length.

- **Flow rate change:**
  In this experiment the test samples were analyzed at the flow rate of 0.8 ml/min and 1.2 ml/min, each and the results were observed in terms of assay value and chromatographic compatibility. Blank, standard and sample solutions were prepared as per assay procedure.
- **Wave length change:**
  In this experiment the test samples were analyzed using different wave length and the results were observed in terms of assay value and chromatographic compatibility. Blank, standard and sample solutions were prepared as per assay procedure.

Ruggedness study

- Ruggedness of the method was evaluated by conducting the assay method by two HPLC systems. Blank, standard and sample preparation as per procedure given on assay.

Table 3: Assay results of ledipasvir and sofosbuvir

	Ledipasvir		Sofosbuvir	
	Standard Area	**Sample Area**	**Standard Area**	**Sample Area**
Injection-1	9610.218	9618.037	929.107	933.278
Injection-2	9596.321	9610.218	898.860	902.356
Injection-3	9610.218	9545.801	909.64	928.769
Injection-4	9596.321	9394.586	898.86	934.043
Injection-5	9578.389	9612.063	891.613	918.958
Average Area	9598.293	9556.141	905.616	923.4808
Assay(%purity)	99.5		101.1	

The amount of ledipasvir and sofosbuvir present in the dosage form was found to be 99.56 % and 101.1 % respectively.

Table 4: Accuracy results of ledipasvir

S.No	% level	Sample area	% recovery	% of mean recovery
1	50	913.199	99.0	
2	50	901.016	98.5	98.7
3	50	929.723	98.7	
1	100	921.96	99.8	
2	100	915.556	99.4	99.6
3	100	931.069	99.6	
1	150	1370.413	100.1	
2	150	1331.217	99.8	100.1
3	150	1336.835	100.2	

Table 5: Accuracy results of sofosbuvir

S.No	% level	Sample area	% recovery	% of mean recovery
1	50	9603.072	99.3	
2	50	9605.652	99.4	98.8
3	50	9533.752	98.6	
1	100	10641.469	99.1	
2	100	10610.711	98.8	99.6
3	100	10641.469	99.1	
1	150	11814.282	97.8	
2	150	11810.235	97.7	100.1
3	150	12769.881	100.1	

Recovery of ledipasvir and sofosbuvir were determined at three different concentration levels. The mean recovery for ledipasvir was 98.7-100.1% and sofosbuvir was 98.8-100.1%. The result indicating that the method was accurate.

Table 6: Robustness study of ledipasvir

Summary of robustness study of ledipasvir				
Robust Condition	**% Assay**	**Retention time(min)**	**System suitability**	
			Theoretical plates	**Asymmetry**
Flow 0.8 ml/min	99.7	3.074	4001	1.001
Flow 1.2 ml/min	100.2	3.087	3287	1.238
220 nm	100.1	2.981	3681	1.138
224 nm	98.3	3.010	3448	1.100

Table 7: Robustness study of sofosbuvir

Summary of robustness study of sofosbuvir				
Robust Condition	**% Assay**	**Retention time(min)**	**System suitability**	
			Theoretical plates	**Asymmetry**
Flow 0.8 ml/min	101.0	7.410	3085	1.431
Flow 1.2 ml/min	98.0	7.511	2779	1.401
220 nm	101.0	7.012	3201	1.400
224 nm	102.0	7.121	3091	1.389

The results of robustness study of the developed assay method was established in table 5 and table 7. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was accordance with that of actual. System suitability parameters were also found satisfactory; hence the analytical method developed is satisfactory.

Table 8: Ruggedness study of ledipasvir and sofosbuvir

	ledipasvir	**sofosbuvir**
System 01	97.1	100.2
System 02	99.9	100.4
%RSD	0.212	0.258

The results of ruggedness study of the developed assay method was given in table 7, It shows there is no change of percentage of assay which was conducted in two systems.

4. Conclusion

The proposed HPLC method provide simple, specific, precise, accurate, and reproducible quantitative analysis for simultaneous analysis of ledipasvir and sofosbuvir in combined dosage form. The method was validated as per ICH guidelines in terms of specificity, linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ), robustness, and reproducibility. The proposed method can be used for routine analysis and quality control assay of ledipasvir and sofosbuvir in combined dosage form.

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