Research Article

New Method Development, Validation and Stability Indicating Studies of Ledipasvir and Sofosbuvir in Bulk and Pharmaceutical Dosage Forms by Using RP-UPLC

Sandhya Rani Baratam[1]*, Srinivasa Rao Atala[2], Ramanamma Lalam[3], Balakrishna Konna[3]

1. *Research Scholar, Sri Vishnu College of Pharmacy, Bhimavaram, AP, India.
2. Professor, Sri Vishnu College of Pharmacy, Bhimavaram, AP, India.
3. Assistant Professor, Raghu college of pharmacy, Visakhapatnam, AP, India.

***Corresponding author's E-mail:** sandhyarani.pharma@gmail.com

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ABSTRACT

A novel and simultaneous stability indicating RP-UPLC method has been developed for quantitative analysis of Ledipasvir and Sofosbuvir in bulk and pharmaceutical dosage forms. RP-UPLC was carried out on AQUITY UPLC BEH C_{18} column (150 mm x 2.1 mm, 2 µm particle size) as a stationary phase and using mobile phase 0.1% Orthophosphoric acid and Methanol. All the compounds are monitored using photodiode array detector at 236 nm with an isocratic method and the flow rate of 1.0 mL/min was maintained. Validation of method was performed 'as per ICH Q1 R2 guidelines. The linearity of the proposed method was found to be 45-135 µg/mL and 200-600 µg/mL /mL respectively for Ledipasvir and Sofosbuvir and its impurities. Therefore, a sensitive, robust, accurate method with high degree of sensitivity was developed for practical utility.

Keywords: Ledipasvir, Sofosbuvir, RP-UPLC, Linearity, Pression, impurity, ICH.

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INTRODUCTION

Ledipasvir

Ledipasvir Fig.1. ((2S)-1-[(6S)-6-[5-(9,9-difluoro -7-{2-[(1R, 3S, 4S)-2-[(2S)-2-{[hydroxyl (methoxy) methylidene] amino}-3-methyl butanoyl]-2-azabicyclo [2.2.1] heptan-3-yl] -1H-1,3 -benzodiazol6-yl}-9H-fluoren-2-yl)-1H-imidazol-2-yl]-5-azaspiro[2.4 heptan-5-yl]-2-{[hydroxyl (methoxy) methylidene] amino}-3-methyl butan-1-one) is a Hepatitis C Virus NS5A Inhibitor with potential activity against HCV.

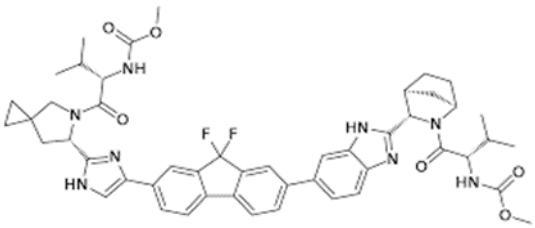


Figure 1: Chemical Structure of Ledipasvir

In combination with sofosbuvir for treatment in chronic hepatitis C genotype 1 patients.[1] Upon oral administration and after intracellular uptake, ledipasvir binds to and blocks the activity of the NS5A protein. 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, plays a crucial role in HCV RNA replication ratio.1.[2]

Sofosbuvir

Sofosbuvir1,2 Fig.2. (Propan-2-yl (2S)-2-{[(S)-{[(3R,4R,5R) -5-(2,4-dioxo-1,2,3,4-tetrahydro pyrimidin1-yl) -4-fluoro-3-hydroxy-4-methyloxolan-2-yl]methoxy phenoxy) phosphoryl] amino} propanoate) is a prodrug nucleotide analog used as part of combination therapy to treat hepatitis C virus (HCV) infection or to treat co-infection of HIV and HCV.[3] It has a molecular formula of C22H29FN3O9P and a molecular weight of 529.45. After metabolism to the active antiviral agent 2'-deoxy-2'-α-fluoro-β-C-methyluridine-5'-triphosphate (also known as GS-461203), the triphosphate serves as a defective substrate for the NS5B protein, an RNA dependent RNA polymerase required for replication of viral RNA.[4]

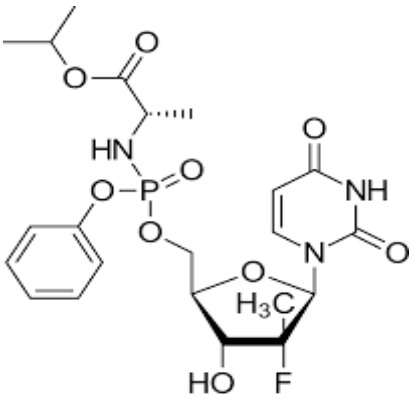


Figure 2: Chemical Structure of Sofosbuvir

Combination of these two drugs (Ledipasvir 90mg + Sofosbuvir 400mg:is available in local pharmacy under the brand name of MyHep LVIR, LEDIFOS TABLET, HETEROSOFIR PLUS and HEPCINAT LP TABLET etc. The mechanism action involves that sofosbuvir inhibits the RNA polymerase that the Hepatitis C virus uses to replicate its RNA and whereas ledipasvir inhibits an important viral phosphoprotein, NS5A, which is involved in viral replication, assembly, and secretion.[5] The present study aimed to develop a simple, sensitive, short retention time and accurate RP-UPLC method for the simultaneous determination of both sofosbuvir and ledipasvir together in pure and tablet dosage forms with high sensitivity, selectivity that can be used for the routine analysis of production samples.[6]

MATERIALS AND METHODS

Drug sample: Ledipasvir 90mg + Sofosbuvir 400mg tablet from spectrum laboratories Hyd.

Mobile phase composition: 0.1% Ortho phosphoric acid and Methanol (45:55)

Instruments Used: UPLC from Waters ACQUITY model

Balance: Digital Balance LC-GC AGN204-PO

pH meter: LABINDIA PICO+

Sonicator: PCI Instruments

Vacuum filtration unit: 0.45 µm membrane filters were obtained from Millipore Pvt. Ltd., Bangalore, India.

Reason for Selection of Mobile Phase

- pH range of Ledipasvir is 2 to 7.7, Sofosbuvir is 3 to 7 and column is 1.5 to 9.
- So, the mobile phase with the pH range of 3 to 7 is more effective.
- pH range of water is 7, methanol is 4.5 to 6 and Ortho phosphoric acid is 5.5 to 7. Hence, these three solvents can be used for the mobile phase
- Both Ledipasvir and Sofosbuvir are freely soluble in aqueous solutions, methanol and insoluble in organic solvents.
- The cost of water and methanol (UPLC grade) is very low when compared with the cost of organic solvents like acetonitrile, ether and acetone etc., and those are easily available in the market. By considering all the above properties, Water: Methanol is the best choice for the mobile phase. A proper ratio of these two will elute sharp peaks which are reproducible with excellent tailing factor.

Detection of wavelength

The sensitivity of the UPLC method depends upon the proper selection of wavelength. Drug solution of 100 µg/mL was scanned over the range of 200-400 nm in UV region using different solvents like water, acetonitrile, hexane and methanol. It was observed that the drug showed maximum absorbance in methanol and water at 236 nm and hence methanol and water was used as solvent and 236 nm was used as maximum wavelength for detection of Ledipasvir and Sofosbuvir for further study.

Preparation of Standard stock solutions

Accuratelyweigh and transfer 90mg of Ledipasvir and 400mg Sofosbuvir into 100mL of volumetric flask and add 10 mL of Methanol and sonicate 10min (or) shake 5min and make with water. Transfers the above solution into 2.5mL into 25mL volumetric flask dilute to volume with water.

Preparation of sample stock solution

Commercially available 20 tablets ware weighed and powdered, the powdered equivalent to the 519.7 mg of Ledipasvir and Sofosbuvir of active ingredients were transfer into a 100mL of volumetric flask and add 10mL of Methanol and sonicate 20min (or) shake 10min and makeup with water.

Preparation of dilutions

Transfers above solution 2.5mL into 25mL of the volumetric flask dilute the volume with Methanol. And the solution was filtered through 0.45µm filter before injecting into UPLC system.

RESULTS AND DISCUSSIONS

Optimized Chromatographic Conditions

Column: AQUITY UPLC BEH C_{18} column (150 mm x 2.1 mm, 2 µm particle size)

Run Time : 8min

Wavelength : PDA- 236 nm

Flow rate :1.0 mL/min

Injection volume :10 µL

Column temp : Ambient

Pump mode : Isocratic

Mobile phase : 0.1% OPA and Methanol (45:55)

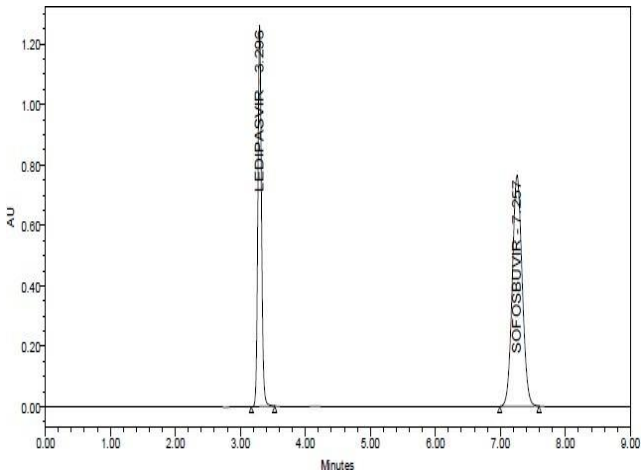


Figure 3: System suitability chromatogram of Ledipasvir and Sofosbuvir

Inference

Peak obtained for both Ledipasvir and Sofosbuvir was good with excellent peak characteristics and it was eluted at 3.296 min and 7.257 min for Ledipasvir and Sofosbuvir respectively. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated.

Method Validation

The optimized method which is derived from the trials can be validated and all the parameters should be checked. The following parameters can be validated in UPLC method.

They are:[3,4,5]

1) System suitability

2} Specificity

3) Linearity

4) Precision

5) Accuracy

6) Sensitivity

7) Robustness

1. System Suitability

All the system suitability parameters were within the range and satisfactory as per ICH guidelines Q1 R2. The results are reported in table 1.

Table 1: System suitability parameters of Ledipasvir and Sofosbuvir

S.No	Retention Time		Tailing Factor		Theoretical Plates	
	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir
1.	3.301	7.301	1.12	1.08	15844	10496
2.	3.308	7.334	1.10	1.08	16253	10564
3.	3.304	7.368	1.09	1.07	16360	10781
4.	3.301	7.392	1.10	1.06	15504	10553
5.	3.304	7.421	1.09	1.06	15520	10416

Inference

According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2. All the system suitable parameters were passed and were within the limits.

2. Specificity

Specificity studies were carried for both pure drugs and drug product by comparing with blank and placebo Table 2. These blank and placebo were compared with standard and sample shows that the analyte chromatographic peak is not attributable to more than one component as the impurities are not available.[8]

Forced Degradation Studies

In the present investigation, as there was no interference of impurities with the analyte peaks, forced degradation studies were conducted with the same LC conditions developed to separate drug peaks of interest from their degradants which proves the stability indicating power of the method.[9] Intentional degradation was attempted to various stress conditions such as acid hydrolysis (using 1.0N HCl), base hydrolysis (using 1.0 N NaOH), oxidative hydrolysis (using 3.0%v/v H_2O_2), thermal degradation (heated at 70°C for 14 days) and photolytic degradation (to overall illumination of ≥210Wh/m^2 at room temperature with UV light for 14 days), to evaluate the ability of the proposed method to separate Ledipasvir and Sofosbuvir from their degradation products.[10,11]

Acidic hydrolysis:

Forced degradation in acidic media was performed by taking accurately weighed samples of 519.7mg of Ledipasvir and Sofosbuvir each in separate 5mL volumetric flask then .[12]2mL of 1N HCl was added, made to dissolve and final volume was made up to the mark with 1N HCl to get mg/mL solutions and these were kept at 70ºC for 2 days and analyzed after suitable dilution.

Basic hydrolysis:

Forced degradation in basic media was performed by dissolving separately an accurately weighed quantities (122.5mg each) of Ledipasvir and Sofosbuvir in 1N NaOH in 5mL volumetric flasks and final volume was made up to 5mL with the same to get mg/mL solutions and these solutions dilution.[13]

Oxidative degradation:

Were kept at 70ºC for 2 days and analyzed after suitable Oxidative degradation studies were carried out in 3% (v/v) H_2O_2. Stock solutions of 122.5mg/mL of Ledipasvir and Sofosbuvir were prepared and kept at 70ºC for 2 days and analyzed after suitable dilution photo.[14] Degradation: For photolytic stress, samples of drug substances in solid state were irradiated with UV

radiation (overall illumination of ≥210Wh/m2 at room temperature with UV radiation), for 14 days. Stock solutions of 1mg/mL were prepared in methanol from the exposed drug substances individually.

Thermal Degradation:

For thermal stress, 122.5mg of samples of drug substances in solid state were packed in glass 1mg/mL were prepared in methanol from the exposed drug substances individually. For UPLC analysis, all the stressed sample solutions were diluted with mobile phase to obtain final concentration of 60µg/mL of Ledipasvir and Sofosbuvir and 100µg/mL of Rabeprazole respectively. Similarly, mixture of both drugs in a concentration of 60µg/mL of Ledipasvir and Sofosbuvir and 100µg/mL of Rabeprazole was prepared prior to analysis by UPLC. [15,16]

Besides, solutions containing 60µg/mL of Ledipasvir and Sofosbuvir and 100µg/mL of Rabeprazole for each drug separately were also prepared without performing the degradation of both the drugs. Then 20µL of above solutions were injected into the UPLC system and analyzed. Solution of standard, sample, blank and placebo were prepared as per test procedure and injected into the UPLC system.[17]

3. Linearity

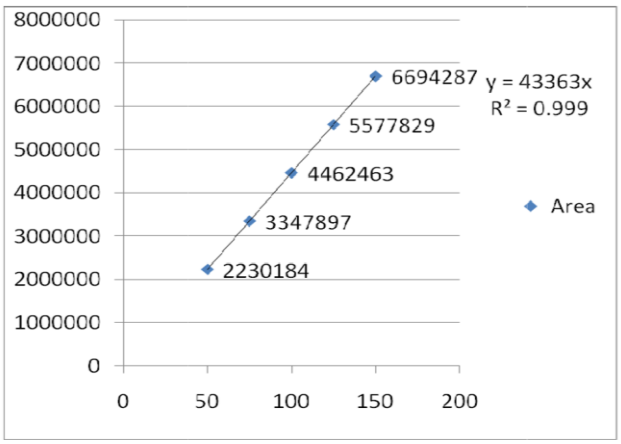
Linearity is the property of a mathematical relationship or function which means that it can be graphically represented as a straight line.[8] Linearity was studied by analyzing five standard solutions covering the range of standard concentrations of sample solutions.

Table 2: Forced degradation study for Ledipasvir and Sofosbuvir

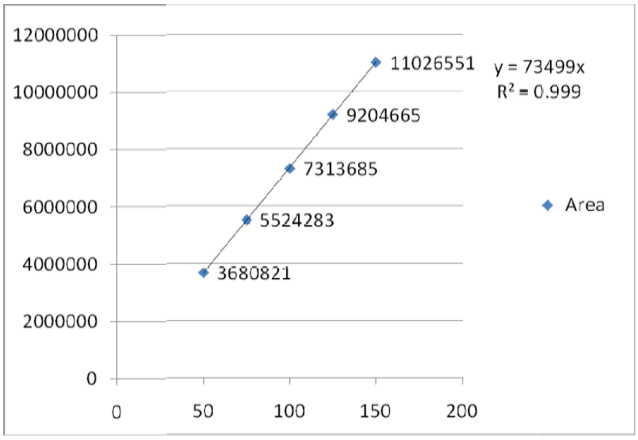
Type of stress	Retention time	% Area	Peak purity	Retention time	% Area	Peak purity	Result
	Ledipasvir			Sofosbuvir			
Acidic Hydrolysis (mg/mL in 1N HCl) at 70ºC for 2 days	3.252	4236590	0.999	6.847	7039509	0.999	Passed
Basic Hydrolysis (mg/mL in 1N NaOH) at 70ºC for 2 days	3.265	4227521	0.999	6.977	7081666	0.999	Passed
Oxidative Hydrolysis (mg/mL in 3% v/v H_2O_2) at 70 ºC for 2 days	3.277	4299323	0.999	7.056	7045562	0.999	Passed
Photo Degradation (to UV light) for 14 days	3.287	4261372	0.999	7.138	7066831	0.999	Passed
Thermal Degradation at 70ºC for 14 days	3.287	4213277	0.999	7.113	7027361	0.999	Passed

Table 3: Linearity data for Ledipasvir and Sofosbuvir

S. No	Ledipasvir			Sofosbuvir		
	Conc (µg/mL)	RT	Area	Conc (µg/mL)	RT	Area
1.	50	3.297	2230184	50	7.253	3680821
2.	75	3.299	3347897	75	7.246	5524283
3.	100	3.297	4462463	100	7.241	7363685
4.	125	3.297	5577829	125	7.228	9204665
5.	150	3.302	6694287	150	7.232	11026551
Correlation coefficient (r^2)			0.999			0.999



Plot 1: Linearity plot of Ledipasvir



Plot 2: Linearity plot of Sofosbuvir

Inference

Retention times of Ledipasvir and Sofosbuvir were found to be 3.296 min and 7.257. We did not find any interfering peaks in blank. So, this method was said to be specific respectively.

4. Precision

Precision is a description of random errors, a measure of statistical variability.7 The Precision of the instrument was checked by repeated injection and measurement of peak areas and retention time of solution.

Inference:

The %RSD of precision were found to be 0.18 for Ledipasvir and 0.12 for Sofosbuvir respectively

5. Accuracy:

Accuracy is the degree of closeness of measurements of a quantity to that quantity's true value. Accuracy of the method was determined by calculating the recoveries of Ledipasvir and Sofosbuvir by the standard addition method.

Table 4: Precision data for Ledipasvir and Sofosbuvir

S. No	Ledipasvir			Sofosbuvir		
	RT	Area	%Assay	RT	Area	%Assay
Injection1	3.320	4465231	99	7.458	7360011	100
Injection2	3.321	4462350	99	7.471	7368755	100
Injection3	3.316	4464645	100	7.451	7364800	100
Injection4	3.312	4462083	99	7.419	7365230	100
Injection5	3.313	4468154	100	7.398	7361573	100
Injection6	3.312	4466897	99	7.370	7361600	100
Mean			99			100
Std. Dev.			0.18			0.12
% RSD			0.18			0.12

Table 5: Accuracy (%recovery) results of Ledipasvir

S.NO	Accuracy Level	Sample name	Sample weight	µg/mL added	µg/mL found	% Recovery	% Mean
1	50%	1	61.25	44.550	44.67	100	100
		2	61.25	44.550	44.76	100	
		3	61.25	44.550	44.73	100	
2	100%	1	122.50	89.100	89.22	101	100
		2	122.50	89.100	89.29	100	
		3	122.50	89.100	89.23	100	
3	150%	1	183.75	133.650	133.82	100	100
		2	183.75	133.650	133.76	100	
		3	183.75	133.650	133.87	100	

Table 6: Accuracy (%recovery) results of Sofosbuvir

S.NO	Accuracy level	Sample name	Sample weight	µg/mL added	µg/mL found	% Recovery	% Mean
1	50%	1	61.25	200.000	199.54	100	100
		2	61.25	200.000	199.51	100	
		3	61.25	200.000	199.71	100	
2	100%	1	122.50	400.000	398.68	100	100
		2	122.50	400.000	398.89	100	
		3	122.50	400.000	398.67	100	
3	150%	1	183.75	600.000	597.91	100	100
		2	183.75	600.000	598.21	100	
		3	183.75	600.000	597.97	100	

Inference

The average percentage recovery was between i98-102% and Relative standard deviation of these recovery concentrations was less than 2%.

6. Sensitivity

a) LOD: It is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) within a stated confidence limit.

LOD = (3.3 x S.D.)/ slope

b) LOQ: It is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met.

LOQ = (10 x S.D.)/ slope

Table 7: LOD and LOQ data for Ledipasvir and Sofosbuvir

Sample name	LOD data		LOQ data	
	RT	Area	RT	Area
Ledipasvir	3.294	1277996	3.298	1523173
Sofosbuvir	7.248	2276040	7.247	2717390

Inference

The LOD value was found to be 0.090 µg/mL for Ledipasvir and 0.090 µg/mL for Sofosbuvir at signal to noise ratio 3:1. LOQ value was found to be 0.301 µg/mL for Ledipasvir and 2.2063 µg/mL for Sofosbuvir at signal to noise ratio 10:1.

7. Robustness

It is the measure of a method remain unaffected by small deliberate changes in method parameters like flow rate and mobile phase composition ratio

Table 8: Robustness data for Ledipasvir and Sofosbuvir

Robustness data for Ledipasvir and Sofosbuvir						
Parameter	RT	Theoretical Plates	Asymmetry	RT	Theoretical plates	Asymmetry
Decreased flow rate (0.8mL/min)	4.127	18129	1.11	9.065	12683	1.07
Increased flow rate (1.2mL/min)	2.741	15685	1.10	6.003	9851	1.07
Decreased temperature (23°C)	3.288	16823	1.09	7.212	10970	1.07
Increased temperature (27°C)	3.266	17367	1.06	6.845	12028	1.04

Inference

The %RSD for flow rate was found to be 1.11 and 1.10 for pregabalin and 1.07 and 1.07 for Methyl cobalamin and %RSD for temperature was found to be 1.09 and 1.06 for Pregabalin and 1.07and 1.04 for Methyl cobalamin respectively.

CONCLUSION

The Ledipasvir and Sofosbuvir showed linearity in the range of 45-135 µg/mL and 200-600 µg/mL respectively. The slope and correlation coefficient values for Ledipasvir were found to be 43363 and 0.999 respectively and 73499 and 0.999 respectively for Sofosbuvir which indicates excellent correlation between response factor Vs co ncentration of standard solutions. Precision of the developed method was studied under system precision and method precision. The %RSD values for precision was found to be within the acceptable limit, which revealed that the developed method was precise. The developed method was found to be robust. The %RSD value for percentage recovery Ledipasvir and Sofosbuvir was found to be within the acceptance criteria. The results indicate satisfactory accuracy of method for simultaneous estimation of the Ledipasvir and Sofosbuvir. The forced degradation study showed the method was highly specific.

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