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Original Article

BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF LEDIPASVIR AND SOFOSBUVIR DRUGS IN HUMAN PLASMA BY RP-HPLC METHOD

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ABSTRACT

Objective: A novel, sensitive and accurate high-performance liquid chromatography with ultraviolet/visible light detection (HPLC-UV/VIS) method for the quantification of ledipasvir and Sofosbuvir in plasma was developed and validated.

Methods: The analytes were extracted by liquid-liquid extraction method and chromatograph using a mobile phase consisting of acetonitrile and buffer solution, Methanol and Acetonitrile in the ratio of 200:600:200 (v/v) using Oyster BDS RP-C18 column. The flow rate 1.0 ml/min and UV detection at 238 nm were employed. The retention time for Ledipasvir and Sofosbuvir was 4.61 and 9.09 min respectively. Linearity for ledipasvir and Sofosbuvir was found to be in the range of 250-2000 ng/ml for both drugs respectively. Intra-and inter-day precision was less than 2% coefficient of variation.

Results: The method was validated as per the USFDA guidelines and the results were within the acceptance criteria for selectivity, sensitivity, linearity, precision, accuracy, recovery stability of the solution, the stability of solution in plasma and dilution integrity.

Conclusion: Majority of the HPLC method should be useful for monitoring human plasma drug concentrations, and pharmacokinetic studies in patients diagnosed with the Ledipasvir and Sofosbuvir formulations.

Keywords: Ledipasvir, Sofosbuvir, Bio-analytical, RP-HPLC, Plasma

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INTRODUCTION

Hepatitis C virus (HCV) one of the major global health problems. HCV infection is responsible for 3,50,000 death cases annually [1]. It is one of the top five death-causing diseases in the country. HCV infection is particularly a national problem in Egypt. The incidence rate reaches 14.5% among Egyptian population, which represents the highest prevalence of hepatitis C worldwide [2]. According to the egyptian ministry of health, 1,00,0000 new cases are identified each year [3]. Of particular note, the first all oral treatments, without the need for Ribavirin or pegIFNa injections, were recently approved: the combination pill, Harvoni TM (Ledipasvir, [4] NS5B inhibitor/ Sofosbuvir 1), a combination of Simeprevir (2) and Sofosbuvir (1) and Viekira PakTM, a combination of three DAAs and Ritonavir, i.e., Paritaprevir [5] (3) (NS3 protease inhibitor), several NS3 protease inhibitors Danoprevir (4), [6] and also Ledipasvir 5 (LED) known as GS-5885, is an NS5A inhibitor and antiviral against HCV (genotypes 1a and 1b)[7] fig. 1).

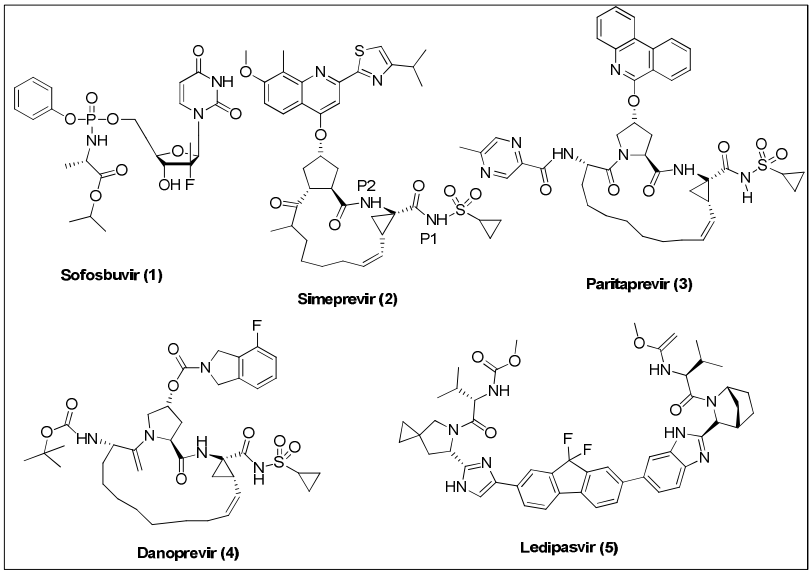

Fig. 1: Examples of approved drugs for HCV treatment: the HCV NS3/4a protease inhibitors 2, 3, 4 and the NS5B, NS5A polymerase inhibitor 1, 5 respectively

Ledipasvir, [methyl ((*S*)-1-((*S*)-6-(4-(9,9-difluoro-7-(2-((1R,3R,4R)-2-((S)-2-((1-methoxyvinyl) amino)-3-methylbutanoyl)-2-azabicyclo [2.2.1]heptan-3-yl)-1H-benzo[d]imidazol-6-yl)-9H-fluoren-2-yl)-1H-imidazol-2-yl)-5-azaspiro [2.4] heptan-5-yl)-3-methyl-1-oxobutan-2-yl) carbamate] is belongs to the class of organic compounds known as fluorenes used for the treatment of hepatitis C [8]. It acts against HCV and is categorized as a direct-acting antiviral agent (DAA). It is an inhibitor of the Hepatitis C Virus (HCV) NS5A protein which is required for viral RNA replication and assembly of HCV virions [9]. Sofosbuvir, IUPAC name is Isopropyl-(2*S*)-2-[[[(2*R*, 3*R*, 4*R*, 5*R*)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetrahydro-furan-2-yl] methoxy-phenoxy-phosphoryl] amino] propanoate, and a nucleotide analog belongs to the class of organic compounds known as pyrimidine 2'-deoxyribonucleosides [10]. It is a pro-drug nucleotide analog used in combination therapy to treat chronic hepatitis C virus (HCV) infected patients with HCV genotype 1,2,3, or 4, and to treat HCV and HIV co-infected patients [11-13]. The combination therapy includes either ribavirin alone or ribavirin and peginterferon alfa. Sofosbuvir prevents HCV viral replication by binding to the two Mg^{2+} ions present in HCV NS5B polymerase's GDD active site motif [14].

Ledipasvir and Sofosbuvir combination, or Ledipasvir in combination with Sofosbuvir and Ribavirin, is indicated for the treatment of chronic hepatitis C (CHC) genotype 1 infection in adults [15]. The fixed-dose combination Ledipasvir-Sofosbuvir (90 mg/400 mg) is indicated for treatment, with or without Ribavirin, for the treatment of patients with chronic hepatitis C genotypes 1, 4, 5, and 6 [16, 17]. Literature states that there are only two analytical methods have been described for analysis of Ledipasvir and Sofosbuvir in an individual by HPLC [18]. Due to high usage of Ledipasvir and Sofosbuvir combination for treatment of hepatitis C the present work is aimed to develop a bio-analytical method for combined analysis of Ledipasvir and Sofosbuvir in plasma.

MATERIALS AND METHODS

Chemicals

Analytically pure drugs were obtained as gift sample reputed pharmaceutical company. Methanol, acetonitrile, water (Merck, Mumbai, India) was of HPLC grade, while potassium dihydrogen phosphate, orthophosphoric acid and triethylamine used for the preparation of mobile phase.

Equipment

Chromatographic separation was performed on a PEAK chromatographic system equipped with LC-P7000 pump, UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Oyster BDS RP-C18 column was used as stationary phase. Teccomp UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Denver electronic analytical balance (SI-234), Systronics digital pH meter was also used.

Preparation pH 4.4 Acetate buffer (USP)

136 g of sodium acetate and 77 g of ammonium acetate are accurately weighed and dissolved in water and dilute to 1000 ml with the same solvent. Then 250.0 ml of glacial acetic acid is added and mixed well to get a buffer solution of pH 4.4.

Preparation of mobile phase

Measure accurately Acetate buffer (pH 4.4) buffer solution, Methanol and Acetonitrile in the ratio of 200:600:200 (v/v) and sonicate the solution for ten minutes mix the contents. The content was mixed and degassed using ultrasonic sonicater, and then it was filtered through 0.45µ nylon membrane filter paper using vacuum filtration set.

Preparation of stock and standard solutions

A stock solution of 1000 mcg/ml of Ledipasvir and Sofosbuvir prepared individually by accurately weighing 100 mg of the standard drugs and was dissolved in 100 ml of methanol to obtain a standard concentration of 1000 mcg/ml. The solutions were filtered and were used as standard stock solutions. From the standard stock solution of 1000 mcg/ml, 1 ml was further diluted to 100 ml to get a working standard solution of 1000 ng/ml. required dilutions were prepared from this working standard stock solution. Aliquots of a standard stock solution of Ledipasvir and Sofosbuvir were transferred using A-grade bulb pipettes into 100 ml volumetric flasks and the solution was made up to volume with methanol to yield a final concentration of 250, 500, 750, 1000, 1250, 1500,1750, 2000 ng/ml individually.

Rinsing solution

7:3 ratios of methanol and Acetonitrile were used as rinsing solution. To this 70 ml of methanol was mixed with 30 ml of acetonitrile in a 100 ml beaker. Mix the solution well and then it was filtered through membrane filter paper. The solution was used as rinsing solution to rinse useful things. The solution was stored at room temperature and used within 7 d from the date of preparation.

Preparation of extraction solution

Diethyl ether and dichloromethane in the ratio of 60:40 (v/v) was used for the extraction of drugs from the biological matrix. 60 ml of Diethyl ether was added to 40 ml of dichloromethane. Mix the solution well and then it was filtered and used for the extraction. The solution was stored at room temperature and used within 7 d from the date of preparation.

Extraction procedure

The liquid-liquid extraction method was used to isolate both the standard drug's plasma. For this, 50 µl of standard drug and 100 µl of plasma sample (respective concentration) were added into labeled polypropylene tubes and vortexed briefly after that 2.5 ml of methyl t-butyl ether was added and vortexed for approximately 10 min followed by centrifuged at 4000 rpm for approximately 5 min at 20 °C. Supernatant from each sample was transferred to labeled vial tube and evaporated at 40 °C until dryness. These samples were reconstituted with 500 µl of reconstitution solution [Diethyl ether and dichloromethane] and vortexed briefly, and then transferred the sample into a clean dry test tube and was used for analysis.

HPLC chromatography conditions

The HPLC isocratic elution was run with mobile phase buffer solution, Methanol and Acetonitrile in the ratio of 200:600:200 (v/v) at pH4.4 and 1 ml/min flow rate. The chromatographic separation was achieved on Oyster BDS RP-C18 5 µm, 250 mm X 4.6 mm i.d. column at 238 nm UV detector wavelength. The column was maintained at room temperature and an injection volume of 20 µl was used. The mobile phase was filtered through 0.45 µm Chrom Tech Nylon-66 filter for use.

RESULTS AND DISCUSSION

One of the most difficult task during the method development was to achieve a high and reproducible recovery from the solvent which is used for extraction of the drug and also difficult task to select such single extracting solvent from which both the drugs are extracted. Different solvents were tried for the extraction of Ledipasvir and Sofosbuvir from human plasma and extraction with methyl t-butyl ether which is reconstituted solution [Diethyl ether and dichloro methane] was exhibited good recovery. Under the optimal conditions (table 1) employed, the retention times were 4.61 min and 9.09 min for Ledipasvir Sofosbuvir respectively, with good peak shape and resolution (table 2, fig. 2, 3). The proposed chromate-graphic conditions are validated according to the ICH and US-FDA guidelines [19-21].

Selectivity and system suitability

The selectivity of the method was evaluated by analyzing six independent drug-free human plasma samples with reference to potential interferences from endogenous and environmental constituents. In optimization, trials choose such method where plasma lots were found to be free of significant interferences. Resolution, tailing factor and theoretical plate's results were with the acceptable limit thus meets the system suitability criteria.

Calibration curve/linearity

The Eight point calibration curve was constructed by plotting the peak response ratio of Ledipasvir and Sofosbuvir in plasma. Correlation of coefficients is 0.999 and 0.998 for Ledipasvir and Sofosbuvir respectively. Linearity's were found over the range 250, 500, 750, 1000, 1250, 15000, 1750, 2000ng/ml for both Ledipasvir and Sofosbuvir. The lower limit of quantification was defined as lowest concentration in the calibration curve. The Ledipasvir and Sofosbuvir can be determined at LLOQ 200ng/ml. Data of calculated calibration standard concentration are shown in table 3 respectively and a representative calibration curve is shown in fig. 4.

Precision and accuracy

The precision of the method was determined by repeatability and accuracy for set of quality control (QC) sample (low, mid, high) in replicate (n = 6). The precision was found to be in the range (% CV) of 0.861-0.580%, 1.388-0.513 and 1.275-0.813% for LQC, MQC and HQC respectively. In this assay the inter-day, intra-day precision and accuracy values were within the acceptable range, it shows that the method is accurate and precise. The low percent relative standard deviation and percent relative error were within the acceptable limit. The results of precision and accuracy for the Ledipasvir and Sofosbuvir are shown in table 4, 5 and 6.

Recovery

Absolute recovery was calculated by comparing peak areas obtained from freshly prepared sample extracted with unextracted standard solutions of the same concentration. Recovery data were determined in triplicates at 750ng/ml. The recovery of Ledipasvir and Sofosbuvir for was found to be 87.467 %, 85.491 respectively (table 7).

Ruggedness and robustness

The ruggedness of the extraction procedure and the chromatographic method was evaluated by analysis at 750ng/ml concentration by a different analyst. Within batch precision of the method was in the range of 101.2 to 102.6 % and 100.4 to 102.3% for Ledipasvir and Sofosbuvir, respectively. Robustness results are achieved in the range of 0.136 to 0.179% and 0.134 to 0.89 % of the change in the results.

Stability

Stabilities of the samples were determined in various phases of the method. The stability studies include stock solution stability, freeze-thaw stability, in-injector stability, bench-top stability and long-term stability. All the above stability studies indicate that the samples in various phases were within the acceptance limits. The concentration of the freeze-thaw samples was found to be 91.9-104.3% of the nominal concentration for Ledipasvir and 91.5-101.2% for Sofosbuvir, indicating the stability of the analytes over three freeze-thaw cycles. For the bench top stability, the back-calculated concentration against freshly spiked calibration standards was found to be 93.6 to 100.1% of the nominal concentration for Ledipasvir and 92.7 to 100.3% Sofosbuvir. The concentration of the long term-stability samples ranged between 87.3 to 99.4% and 84.8 to 98.9% of the nominal value, respectively, for Ledipasvir and Sofosbuvir. The long-term stability duration was calculated as the date of analysis of QC samples, less the date of preparation of the stability QC samples.

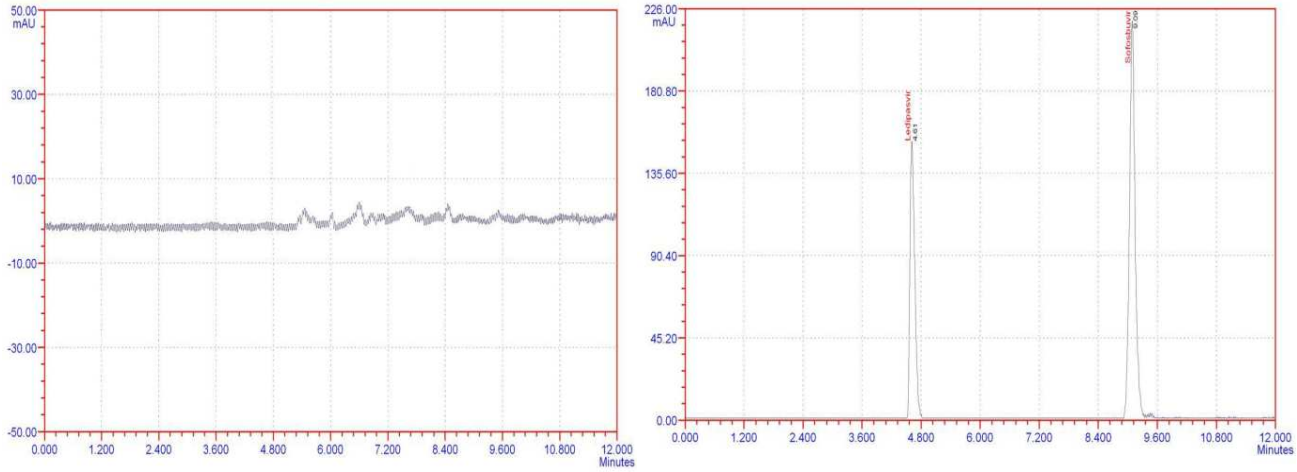

Fig. 2: Blank and standard chromatograms of ledipasvir and sofosbuvir

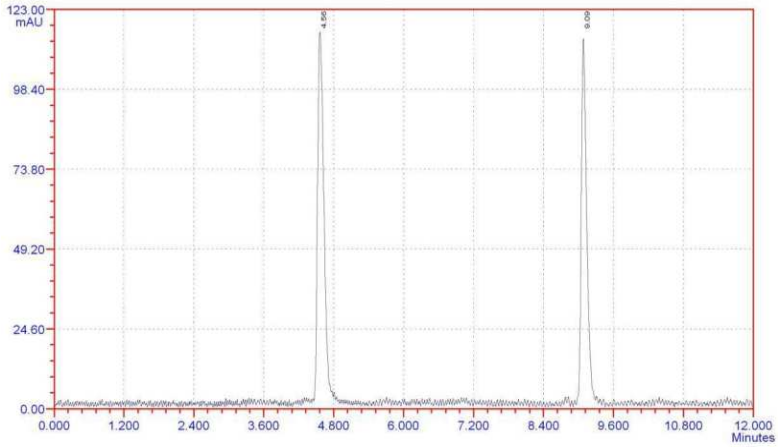

Fig. 3: Sample chromatograms of Ledipasvir and Sofosbuvir

Table 3: Plasma spiked calibration curve results

Test	Sample ID	Ledipasvir		Sofosbuvir	
		Concentration prepared	Area obtained	Concentration prepared	Area obtained
PSCC	PSCC01	250ng/ml	52563	250ng/ ml	63342
	PSCC02	500ng/ml	80925	500ng/ ml	108016
	PSCC03	750ng/ml	104220	750ng/ ml	154431
	PSCC04	1000ng/ml	134956	1000ng/ ml	210252
	PSCC05	1250ng/ml	157489	1250ng/ ml	252746
	PSCC06	1500ng/ml	187484	1500ng/ ml	299062
	PSCC07	1750ng/ml	209997	1750ng/ ml	347868
	PSCC08	2000ng/ml	239272	2000ng/ ml	409237
N		8		8	
Slope		105.9		195.0	
Intercept		26686		11142	
r^2		0.999		0.998	

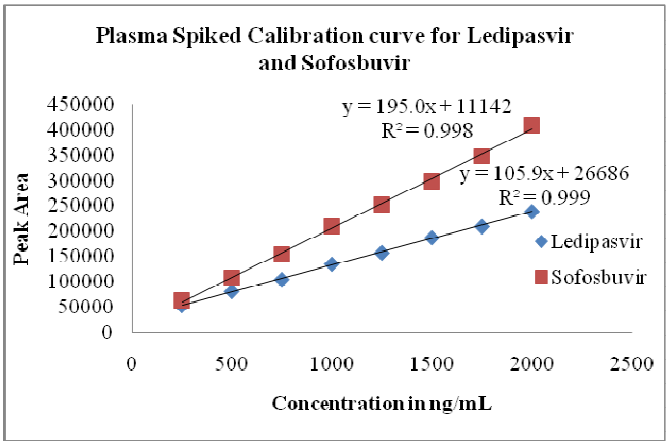

Fig. 4: Plasma spiked calibration graph

Table 4: Results of precision and accuracy at LQC

P and A at LQC					
S. No.	Sample ID	Ledipasvir		Sofosbuvir	
		Area obtained	% accuracy	Area obtained	% accuracy
P and A at LQC	PA001	52326	99.54911	63761	100.6615
	PA002	51615	98.19645	62699	98.98488
	PA003	52802	100.4547	63423	100.1279
	PA004	52324	99.54531	63184	99.75056
	PA005	52814	100.4775	63575	100.3678
	PA006	52648	100.1617	63356	100.0221
Nominal Conc.		250ng/ml		250ng/ml	
N		6		6	
Average		451.488	0.859	367.297	0.580
SD		52421.5	99.731	63333	99.986
%CV		0.861	0.861	0.580	0.580
Accuracy (%)		99.731		99.986	

Table 5: Results of precision and accuracy at MQC

P and A at MQC					
S. No.	Sample ID	Ledipasvir		Sofosbuvir	
		Area obtained	% accuracy	Area obtained	% accuracy
	PA007	104220	100	154431	100
P and A at MQC	PA008	104907	100.6592	155014	100.3775
	PA009	103429	99.24103	156648	101.4356
	PA010	101356	97.25197	154638	100.134
	PA011	102246	98.10593	155211	100.5051
	PA012	104786	100.5431	155582	100.7453
Nominal Conc.		750ng/ml		750ng/ml	
N		6		6	
Average		1437.054	1.379	795.871	0.515
SD		103490.7	99.300	155254	100.533
% CV		1.388	1.388	0.513	0.513
Accuracy (%)		99.300		100.533	

Table 6: Results of precision and accuracy at HQC

P and A at HQC					
S. No.	**Sample ID**	**Ledipasvir**		**Sofosbuvir**	
		Area obtained	**% Accuracy**	**Area obtained**	**% Accuracy**
	PA013	236257	98.73993	407011	99.45606
P and A at HQC	PA014	235011	98.21918	407428	99.55796
	PA015	235109	98.26014	405325	99.04407
	PA016	237572	99.28951	407267	99.51862
	PA017	238177	99.54236	407836	99.65766
	PA018	229776	96.03129	399179	97.54226
Nominal Conc.		2000ng/ml		2000ng/ml	
N		6		6	
Average		2999.594	1.254	3297.963	0.805881
SD		235317	98.347	405674.3	99.129
%CV		1.275	1.275	0.813	0.813
Accuracy (%)		98.347		99.129	

Table 7: Results of plasma spiked recovery

Plasma spiked recovery					
Test	**Sample ID**	**Ledipasvir**		**Sofosbuvir**	
		Area obtained	**% recovery**	**Area obtained**	**% recovery**
	PSR001	107387	87.01222	153329	84.71964
PSR at MQC	PSR002	108486	87.9027	155246	85.77885
	PSR003	108193	87.66529	154568	85.40423
	PSR004	107575	87.16455	155693	86.02584
	PSR005	107795	87.34281	154431	85.32854
	PSR006	108252	87.7131	155087	85.691
Nominal Conc.		750ng/ml		750ng/ml	
N		6		6	
Average		428.753	0.347	824.549	0.456
SD		107948	87.467	154725.7	85.491
%CV		0.397	0.397	0.533	0.533
Recovery (%)		87.467		85.491	

CONCLUSION

In the proposed study, the sensitive isocratic RP-HPLC method has been developed for simultaneous analysis of Ledipasvir and Sofosbuvir in plasma. The developed method was validated and was found to be novel, simple, sensitive, and precise. As the precision accuracy and robustness are a concern the % RSD is less than 2 which is within range of ICH guidelines. Since, for preparation of plasma samples, the developed method involves direct estimation (precipitation of plasma protein by organic solvents) which is simple, cheap, accurate and easy in comparison to solid phase extraction or liquid-liquid extraction. So this HPLC method should be useful for monitoring plasma drug concentrations, and pharmacokinetic studies in patients diagnosed with the Ledipasvir and Sofosbuvir formulations.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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