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

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ABSTRACT

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. The analytes were extracted by liquid–liquid extraction methodand 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-1 and UV detection at 238 nm was employed. The retention time for ledipasvir and sofosbuvir was 4.61 and 9.09 min respectively. Linearity for ledipasvir and sofosbuvirwas found to be in the range of 250-2000ng/ml for both drugs respectively. Intra- and inter-day precision were less than 2% coefficient of variation. 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 solution, stability of solution in plasma and dilution integrity.

KEY WORDS: Ledipasvir, Sofosbuvir, Bioanalytical, RP-HPLC, Plasma

INTRODUCTION

Ledipasvir is belongs to the class of organic compounds known as fluorenes used for the treatment of hepatitis C [1]. 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 [2].Sofosbuvir is a nucleotide analog belongs to the class of organic compounds known as pyrimidine 2'-deoxyribonucleosides [3].It is a prodrug nucleotide analog used in combination therapy to treat chronic hepatitis C virus (HCV) infected patients with HCV genoptype 1,2,3, or 4, and to treat HCV and HIV co-infected patients [4-7]. The combination therapy includes either ribavirin alone or ribavirin and peg-interferon alfa. Sofosbuvir prevents HCV viral replication by binding to the two Mg2+ ions present in HCV NS5B polymerase's GDD active site motif [7].

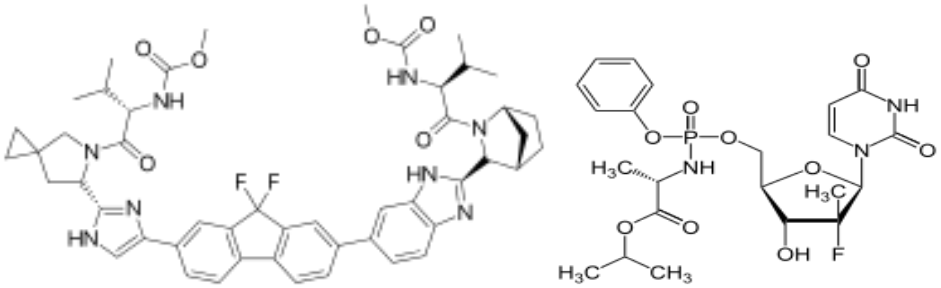

Figure A: Chemical structure of Ledipasvir andSofosbuvir

Ledipasvir and sofosbuvir combination, or Ledipasvirin combination with sofosbuvir and ribavirin, is indicated for the treatment of chronic hepatitis C (CHC) genotype 1 infection in adults [8].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 [9-11]. Literature states that there are only two analytical methods have been described for analysis of Ledipasvir and Sofosbuvir in individual by HPLC [12, 13]. Due to high usage of Ledipasvir and Sofosbuvir combination for treatment of hepatitis C the present work is aimed develop a bioanalytical method for combined analysis of Ledipasvir and Sofosbuvir in plasma.

Experimental

Chemicals and Materials

Analytically pure drugs were obtained as gift samplereputed Pharmaceutical company. Methanol, acetonitrile, water (Merck, Mumbai, India) was of HPLCgrade, while potassium dihydrogen phosphate, orthophosphoricacid 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 were 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 1000ml 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

Stock solution of 1000mcg/ml of Ledipasvir and Sofosbuvir prepared individually by accurately weighing 100mg of the standard drugs and was dissolved in 100ml of methanol to obtain standard concentration of 1000mcg/ml. The solutions were filtered and were used as standard stock solutions. From the standard stock solution of 1000mcg/ml, 1ml was further diluted to 100ml to get a working standard solution of 1000ng/ml. required dilutions were prepared from this working standard stock solution.Aliquots of standard stock solution of Ledipasvir and Sofosbuvir were transferred using A-grade bulb pipettes into 100ml volumetric flasks and the solution were made up to volume with methanol to yield 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 70ml of methanol was mixed with 30ml of acetonitrile in a 100ml 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 days 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 40ml 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 days from the date of preparation.

Extraction procedure: The liquid–liquid extraction method was used to isolate both the standard drugs 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 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 1ml/min flow rate. The chromatographic separation was achieved on a Oyster BDS RP- C18 5µm, 250mm X 4.6mm i.d. column at 238nm UV detector wavelength. The column was maintained at room temperature and injection volume of 20µl was used. The mobile phase was filtered through 0.45µm Chrom Tech Nylon-66 filter for use.

Table 1: Optimized chromatographic conditions:

Parameter	Results
MP	buffer solution, Methanol and Acetonitrile in the ratio of 200:600:200 (v/v)
Wavelength	238nm
Stationary Phase	Oyster BDS RP- C18
pH of MP	4.4
Flow Rate	1.0ml/min
Pump Mode	Isocratic
Run Time	12min
Pump Pressure	10.5±7MPa

Table 2: System suitability results

Parameter	Results
Api Concentration	Ledipasvir – 750ng/ml
	Sofosbuvir - 750ng/ml
RT	Ledipasvir – 4.61min
	Sofosbuvir – 9.09min
Resolution	Ledipasvir – …….
	Sofosbuvir – 14.3
Area	Ledipasvir – 104220
	Sofosbuvir -154431
Theoretical Plates	Ledipasvir – 9117
	Sofosbuvir -30242
Tailing Factor	Ledipasvir – 1.42
	Sofosbuvir – 1.09

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 dichloromethane] was exhibited good recovery. Under the optimal conditions (table.1) employed, the retention times were 4.61min and 9.09min for Ledipasvir Sofosbuvir respectively, with good peak shape and resolution (table.2)(Fig.B, C).The proposed chromatographic conditions are validated according to the ICH and US-FDA guidelines (14-16).

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 plates 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 coeffients is 0.999 and 0.998 for Ledipasvir and Sofosbuvir respectvely. 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 representative calibration curve is shown in figuresD.

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 was determined in triplicates at 750ng/ml. The recovery of Ledipasvir and Sofosbuvir for was found to be 87.467%, 85.491 respectively (Table 7).

Precision and Accuracy

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%, 1.388-0.513and 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.

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 change in the results.

Stability

Stabilities of the samples were determined in various phases of method. The stability studies include stock solution stability, freeze-thaw stability, in-injector stability, bench-top stability and longterm 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 were 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.

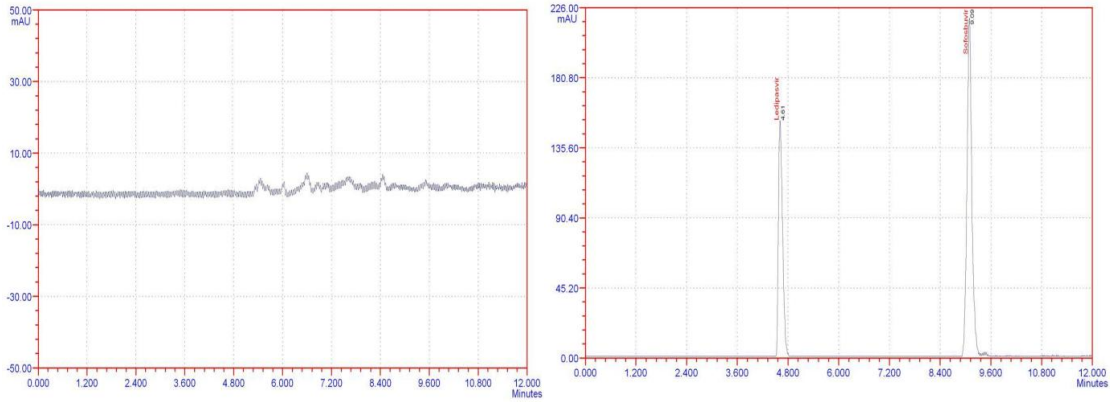

Figure B:blank and standard chromatograms of Ledipasvir and Sofosbuvir

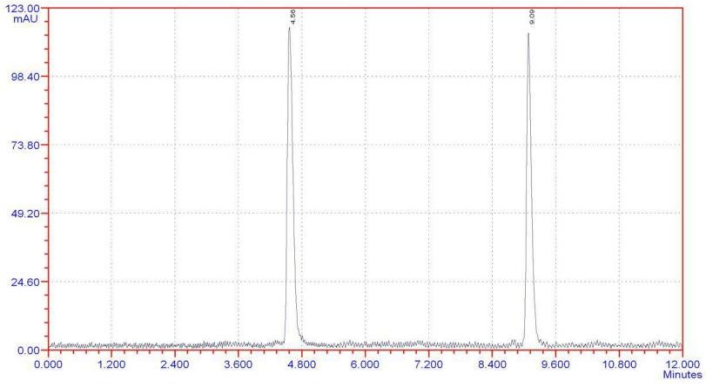

Figure C: sample chromatograms of Ledipasvir and Sofosbuvir

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**	

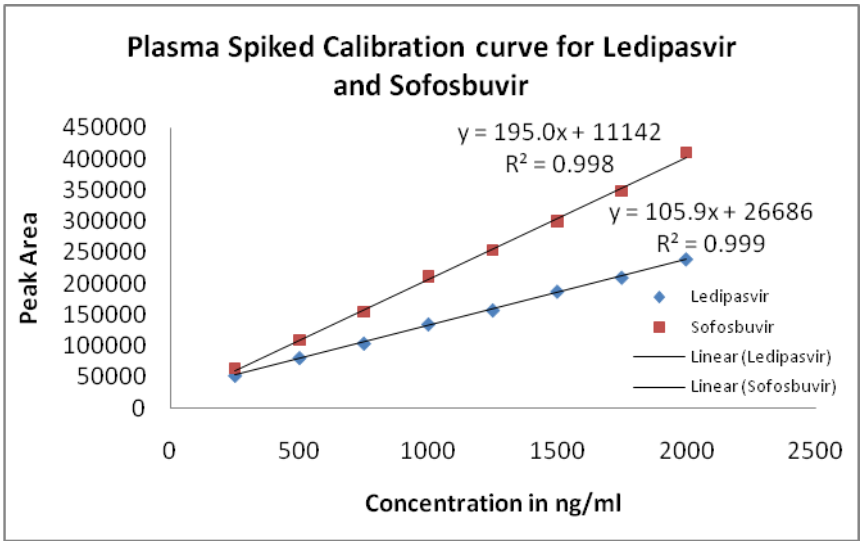

Figure D:Plasma Spiked calibration graph

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
	PA001	52326	99.54911	63761	**100.6615**
P and A	PA002	51615	98.19645	62699	**98.98488**
at LQC	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	PA008	104907	100.6592	155014	**100.3775**
at MQC	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					
		Ledipasvir		Sofosbuvir	
S.NO	Sample ID	Area obtained	% Accuracy	Area obtained	% Accuracy
	PA013	236257	98.73993	407011	**99.45606**
P and A	PA014	235011	98.21918	407428	**99.55796**
at HQC	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					
		Ledipasvir		Sofosbuvir	
Test	Sample ID	Area obtained	% Recovery	Area obtained	% Recovery
	PSR001	107387	87.01222	153329	**84.71964**
PSR at	PSR002	108486	87.9027	155246	**85.77885**
MQC	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 proposed study, 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 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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