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

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ABSTRACT

For the quantification of Sofosbuvir and Ledipasvir in human plasma, A new Reverse Phase High Performance Liquid chromatographic method was developed. A two step simple liquid-liquid extraction (LLE) procedure was carried out for the extraction of Sofosbuvir and Ledipasvir from plasma samples.The developed method resulted in retention times of sofosbuvir and Ledipasvir were found out to be 2.9 and 6.3 min respectively. According to the ICH guidelines, the method was validated with respect to specificity, precision, accuracy and linearity and stability studies. The proposed method was found to be reproducible and convenient for the quantitative analysis of Ledipasvir and Sofosbuvir in bulk form.

KEYWORDS: Sofosbuvir, Ledipasvir, ICH, HPLC.

INTRODUCTION:

This study was pointed towards the build up and validation of a simple methodology to quantify the most used drugs sofosbuvir and ledipasvir for the treatment of hepatitis C virus (HCV) infection[1,2,3,4], in human plasma as per ICH guidelines.[5]Sofosbuvir is prodrug. It is metabolized to the active antiviral agent GS-461203. GS-461203 serves as defective substrate for the NS5B protein, which is the viral RNA polymerase, thus acts as an inhibitor of viral RNA synthesis. [6,7]Ledipasvir is also used for the treatment of hepatitis c virus infection. Ledipasvir inhibits an important viral phosphoprotein, NS5A, which is involved in the viral replication, assembly, and secretion. The combination of sofosbuvir (SBR) and ledipasvir (LDR) is approved by FDA in 2014 for the treatment of HCV. [8,9,10,11,12,13,14,15,16]After literature survey, we have found many chromatographic methods for the determination of sofosbuvir (SBR) and ledipasvir (LDR) by using RP-HPLC.

METHODS:

INSTRUMENTS:

HPLC –SHIMADZU, Model: Prominence Liquid Chromatogram, SPD- 20A UV – VIS detector, wavelength 270nm, Colum Temperature is ambient.

Chromatographic separation was performed on cooling centrifuge C24 REMI centrifuged at 5000 rpm, 4^0C for 20 min.

CHEMICALS:

The drugs sample of sofosbuvir and ledipasvir from A Gift pack from Hetero Drugs. All chemicals and reagents used were of high quality, purity procured from various sources, Acetonitrile, Methanol, Water from Merck (HPLC- Grade), 0.05%Acetic acid from Merck (AR grade). The Optimized conditions for chromatograph are listed in Table No 1.

Preparation of Sofosbuvir Standard Solution:

Weighed down 10mg of Sofosbuvir and transferred it into a volumetric flask. It was dissolved by using 10ml of Methanol and sonicated for 10 minutes to get 1000ppm and 0.1 ml was taken from the solution into a 10ml volumetric flask and diluted to 10 ml with methanol.

Preparation of Ledipasvir Standard Solution:

Weighed down 10mg of Ledipasvir and transferres it into a volumetric flask. It was dissolved by using 10ml of Methanol and sonicated for 10 minutes to get 1000ppm and 0.1 ml was taken from the solution into a 10ml volumetric flask and diluted to 10 ml with methanol.

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Preparation of standard solutions:

The stock solutions of Sofosbuvir and Ledipasvir were again diluted with diluent to produce a series of standard mixtures with final concentration ranging from 1000 – 5000ng/mL respectively. A 1:1 standard mixture containing 1000ng/mL of Sofosbuvir and Ledipasvir were also prepared with the diluent.

Sample preparation:

A two step simple **liquid-liquid extraction (LLE)** procedure was carried out for the extraction of Sofosbuvir and Ledipasvir from plasma samples. To a prepared series of 500µL of drug solutions, 200µL of plasma, acetonitrile were added and mixed for 2 min for de protonation and centrifuged at 5000 rpm, 4^0C for 20 min. The organic layer was separated. From this, required amount was taken and diluted to 10 ml with methanol solution andthen it was injected into HPLC system.

RESULTS

Validation of the Method:

According to ICH guidelines the method was validated, in terms of specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness of the sample.

System suitability:

It was assessed by sx replicate analysis of drug at a concentration of 2000ng/ml of sofosbuvir and ledipasvir.The acceptance criteria are %RSD is not greater than 2.

Selectivity:

The analysis of blank and standard samples is helpful in determining the selectivity. The blank sample was tested for interference and the selectivity was ensured at a lower limit of quantification (LLOQ).

Linearity:

5-point calibration curve were prepared on single day for all methods,. The obtained results were used to calculate the equation of the line using linear regression by the least square method.

Accuracy:

The accuracy of an analytical method is involved in describing the closeness of mean test results obtained from the method to the true value (concentration) of analyte.

Recovery:

The recovery was determined by comparing the aqueous solution and the spiked drug concentrations. Recovery experiments were performed by comparing the analytical results for extracted samples at three concentrations (LQC, MQC, and HQC) with unextracted standards which were used to represent 100% recovery. The results were summarized in table 10&11.

Precision:

The precision of an analytical method is helpful in describing the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous biological matrix volumes.The results were tabulated in Table 6&7.

DISCUSSION

The linearity range of Sofosbuvir and Ledipasvir were evaluated by concentrations ranging from 1000 – 5000ng/mL of extracted plasma sample solutions which were injected into HPLC system. The linearity graphs were plotted from (Fig: 3-4). A relevant calibration curve was made using concentration on x-axis and area under

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the curve on y-axis. The linearity range was found from 1000-5000ng/ml. The regression equation of curve was computed. The correlation coefficient for the data was found as 0.999. The regression line was observed to be in the form of y = 6.2312x + 4.6 for Sofosbuvir. The regression line was observed to be in the form of y = 3.3223x – 281for Ledipasvir. Linearity data for Sofosbuvir and ledipasvir were presented in Table no 2&3.

Accuracy of the method was expressed in terms of recovery of added compounds at 50%, 100% and 150% level of sample. As in the standard preparation, the samples were spiked to the plasma and it was extracted and collected in vials and injected into HPLC system. Mean % recovery and % RSD were calculated and were summarized in Table 4&5. The result shown that the best recoveries (99.77±0.04) of the spiked drug were obtained at each added concentration, indicating the accuracy of a method.

The % Relative standard deviations of Ledipasvir and Sofosbuvir for Repeatability was found to be 1.41 and 1.14. Hencethe %RSD values indicate a good degree of the precision within the specified range.

The % Relative standard deviations of Ledipasvir and Sofosbuvir for Intermediate precession was found to be 1.04 and 1.26.Hence the %RSD values indicate a good degree of the precision within the specified range.

Stability Studies:

Freeze and Thaw Stability:

Analyte stability was determined after three cycles of freeze and thaw. Three of LQC and MQC were stored at the required temperature for about 24 hours and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for about 12 to 24 hours under the same conditions. The freeze–thaw cycle was repeated for two more times and then analyzed on the third cycle.

Short-Term Temperature Stability:

Three aliquots of LQC and HQC were thawed at room temperature and kept at the desired temperature for about 22 hours and analyzed.

Long-Term Stability:

The storage time in the evaluation of long term stability should exceed the time between the date of first sample collection and the date of last sample analysis. So, long-term stability was determined by storing three aliquots of LQC and HQC under the same conditions as the study samples for about 22 days.

Stock Solution Stability:

The stability of stock solutions of drug was evaluated at room temperature for 6 hours. Stability sample results should be within 15% of nominal concentrations.

Parameters	Method
Stationary phase (column)	Zodiac ODS C18(250 x 4.6 mm, 5 µ)
Mobile Phase	Acetonitrile: Methanol: water (60:20:20)
Flow rate (ml/min)	1.0 ml/min
Run time (minutes)	10 min
Column temperature (°C)	Ambient
Volume of injection loop (l)	20
Detection wavSelength (nm)	275nm
Drug RT (min)	2.9 min for sofosbuvir and 6.2 min for ledipasvir

Table No.1: OPTIMATION CONDITIONS

.S.No	Linearity Level	Concentration (ng/mL)	Area
1	I (LQC)	1000	7078
2	II	2000	12747
3	III(MQC)	3000	17513
4	IV	4000	23091

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5	V(HQC)	5000	33062
Correlation Coefficient			0.986

Table no:2. Linearity data for Sofosbuvir.

S.No	Linearity Level	Concentration(ng/mL)	Area
1	I (LQC)	1000	3583
2	II	2000	6334
3	III(MQC)	3000	9167
4	IV	4000	11961
5	V(HQC)	5000	17381
Correlation Coefficient			0.985

Table no:3. Linearity data for Ledipasvir.

Sample ID		Concentration (ng/mL)		%Recovery	Statistical Analysis
		Amount added (ng)	Amount found (ng)		
50 %	**1**	500	482.5	103	**Mean** = 100
	2	500	513.5	102.7	
	3	500	476.5	94.5	
100%	**1**	1000	966.5	96.65	**Mean** = 97.41
	2	1000	980.	98.096	
	3	1000	975.5	97.5	
150%	**1**	1500	1460	97.3	**Mean** = 97.6
	2	1500	1480	98.6	
	3	1500	1456	97	
					Avg =98.33

Table no:4. Accuracy at LLOQ - data for Sofosbuvir.

SampleID		Concentration (ng/mL)		%Recovery	Statistical Analysis
		Amount added (ng)	Amount found (ng)		
50 %	**1**	500	487	97.4	**Mean** = 98.83
	2	500	508	101	
	3	500	489	97.8	
100 %	**1**	1000	970.2	97.02	**Mean** = 97.87
	2	1000	980.6	98.6	
	3	1000	980	98	
150 %	**1**	1500	1440	.96	**Mean** = 97.4

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	2	1500	1480	98.6	
	3	1500	1464	97.6	
					Avg=98.03 %

Table no:5. Accuracy at LLOQ - data for Ledipaasvir.

Precision at LLOQ:

Injection	**Peak area**	
	S	**L**
Injection-1	7078	3583
Injection-2	6923	3548
Injection-3	7096	3672
Injection-4	7067	3513
Injection-5	6952	3592
Injection-6	6966	3517
Average	7012	3570.83
Standard Deviation	179.44	59.32
%RSD	1.05	1.6

Table no:6. Precision at LLOQ data for Sofosbuvir and Ledipasvir.

Injection	**Inter day Peak area**	
	S	**L**
Injection-1	17513	9167
Injection-2	17431	9364
Injection-3	17494	9342
Injection-4	18231	9228
Injection-5	17723	9018
Injection-6	17612	9332
Average	17667	9241.8
Standard Deviation	353	71.16
%RSD	1.98	0.77

Injection	**Intra-day Peak area**	
	S	**L**
Injection-1	18143	9199
Injection-2	18165	9230
Injection-3	18029	9329
Injection-4	18012	9453
Injection-5	18130	9332
Injection-6	18167	9234
Average	18107.66	9296.16
Standard Deviation	465.89	94.49
%RSD	0.38	1.01

Table no:7. Inter-day & Intra-day Precision data for Sofosbuvir and Ledipasvir.

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SampleID	Concentration (ng/mL)		%Recovery	Statistical Analysis
	Amount added (ng)	Amount found (ng)		
LQC	1000	986.6	98.6	**Mean** = 98.42
LQC	1000	989.8	98.98	
LQC	1000	977.3	97.7	
MQC	3000	3000	100	**Mean** = 96.4
MQC	3000	2858.15	95.2	
MQC	3000	2821.19	94.0	
HQC	5000	5000	100	**Mean** = 102.11
HQC	5000	5190	103.8	
HQC	5000	5127.4	102.54	98.97%

Table no:8. Accuracy data of Sofosbuvir.

Sample ID	Concentration (ng/mL)		%Recovery	Statistical Analysis
	Amount added (ng)	Amount found (ng)		
LQC	1000	982	98.9	**Mean** = 97.16
LQC	1000	969.43	96.94	
LQC	1000	956.42	95.64	
MQC	3000	2942.5	98.08	**Mean** = 97.73
MQC	3000	2983.5	99.4	
MQC	3000	2872.4	95.73	
HQC	5000	5000	100	**Mean** = 99.11
HQC	5000	4897.78	97.9	
HQC	5000	4972.4	99.44	98.03%

Table no:9. Accuracy data of Ledipasvir.

Sample ID	Concentration (ng/mL)		%Recovery	Statistical Analysis
	Amount added (ng)	Amount found (ng)		
LQC	1000	897.43	89.74	**Mean** = 91.70%
MQC	3000	2762.4	92.08	

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HQC	5000	4686.4	93.3	

Table no:10. Recovery data for Sofosbuvir.

Sample ID	Concentration (ng/mL)		%Recovery	Statistical Analysis
	Amount added (ng)	Amount found (ng)		
LQC	1000	864.18	86.418	**Mean** = 89.26%
MQC	3000	2820	94	
HQC	5000	4569	87.41	

Table no:11. Recovery data for Ledipasvir.

Name of the drug	Amount added	Amount found (ng)	% Change
Sofosbuvir	LQC (1000 ng)	986	1.4
		974	2.6
		984	1.6
	MQC (3000 ng)	2895	3.5
		2942	1.9
		2924	2.52
Ledipasvir	LQC (1000 ng)	972.5	2.75
		986.5	1.35
		973	2.7
	MQC (3000 ng)	2923	2.56
		2913	2.9
		2906	3.13

Table no:12. Freeze and Thaw Stability data.

Name of the drug	Amount added	Amount found	% Change
Sofosbuvir	LQC (1000ng)	966.6	0.625
		974.8	2.52
		976.4	2.36
	MQC (3000 ng)	2924	1.52
		2899	2.02
		2989	0.22
Ledipasvir	LQC (1000ng)	989.9	1.01
		976.4	2.36
		986.4	1.36
	MQC (3000 ng)	2912	1.76
		2811.5	3.78
		2809.4	3.812

Table no:13. Short-term temperature stability data.

Name of the drug	Amount added	Amount found (ng)	% Change
Sofosbuvir	LQC (1000 ng)	984.9	1.51
		965.4	3.46
		985	1.5
	MQC (3000 ng)	2942	2.5
		2895	3.5
		2924	1.52
Ledipasvir	LQC (1000 ng)	992	0.8
		978	2.2
		986	1.4
	MQC (3000 ng)	2911	2.96
		2963	1.2
		2972	0.93

Table no:14. Long-term temperature stability data

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Name of the drug	Amount added	Amount found	% Change
Sofosbuvir	1000 µg	995 µg	0.5 %
Ledipasvir	1000 µg	997µg	0.79 %

Table no:15. Stock solution stability data.

Conclusions

A simple Bioanalytical method was developed to quantify sofosbuvir& ledipasvir in human plasma. The bio analytical HPLC method was developed by using (ODS) C18 (4.6 x 250mm, 5um, Make: Hypersil) column within a runtime of 10 min. Samples were prepared by using protein precipitation method for analysis. The mobile phase used here was Acetonitrile: Methanol: water (60:20:20)at a flow rate of 1 mL/ min. The mobile phase used for the developing the method was simple to prepare and economical. The % mean recovery was found to be in the range of 89.7-93.3% [sofosbuvir] &86.80–92.5% [ledipasvir].The theoretical plates for Sofosbuvir and Ledipasvir were found to be more than 2000 and the tailing factor is not more than 2.0. The precision of the System and Method were checked and found within limits. This indicates the precise method. The accuracy studies found that the recovery value of pure drug and sample lied between 99.97 % to 100.04% which indicates the accurate method .

The developed method was simple, selective, precise, accurate and rapid. Sofosbuvir& ledipasvir were found to be stable under different stability conditions. The suggested extraction procedure is considerably more simple, rapid, reliable and sensitive when compared to previously published methods. Simple sample preparation procedure and a relatively short chromatographic time make this method more suitable for processing of multiple samples in a limited time for pharmacokinetic studies. This method met the validation criteria laid down by ICH. Hence the developed method can be applied to pharmacokinetics studies and therapeutic drug monitoring in humans.

LIST OF ABBREVIATIONS

FDA - U.S.Food and Drug Administration
RPLC- Reverse Phase liquid chromatography
HILIC- Hydrophilic interaction liquid chromatography,
LC-MS- Liquid chromatography and mass spectroscopy.
RARs - Retinoic acid receptors
IS- Internal Standard
HPLC- High performance liquid chromatography
RIA- Radio immuno assay
ICH- International Council for Harmonisation
LLOQ- Lower limit of Quantification
ULOQ- Upper Limit of Quantification

REFERENCES

1. ICH Guideline on Validation of analytical procedures: text and methodology; Q2 (R1),2005.
2. ICH Guideline on Impurities in new drug products; Q3B (R2),2006.
3. Sharma BK. Instrumental methods of chemical analysis, Introduction to analytical chemistry, 23th ed.Goel publishing house Meerut, 2004, P12-23.
4. P.D. Sethi. HPLC: Quantitative analysis pharmaceutical formulations, CBS Publishers and distributors, New Delhi (India), 2001, P.3-137.
5. https://en.wikipedia.org/wiki/Sofosbuvir
6. https://en.wikipedia.org/wiki/Hepatitis_C
7. https://en.wikipedia.org/wiki/Ledipasvir
8. Bakht zaman, et al, RP-HPLC method for simultaneous determination of sofosbuvir and ledipasvir in tablet dosage form its applications to invitro dissolution studies. Springer link, chromatographia2016, 79, 23-24.
9. Devilal, et al, New method development and validation for the determination of ledipasvir in bulk drug form by Using RP-HPLC. World journal of pharmacy and pharmaceutical sciences2016, 5(8), 1312-1321.
10. Kranthi k k, et al, A new analytical method development and validation for the simultaneous

ISSN:0975-3583,0976-2833 VOL12,ISSUE05,2021

estimation of ledipasvir and sofosbuvir using RP-HPLC. Icjpir2017, 4(1), 142-165.

11. Yogendrachari, et al, Analytical method development and validation for simultaneous determination of ledipasvir and sofosbuvir in tablet dosage form by RP-HPLC. J Global Trends Pharm Sci2016, 7(3), 3386-3393.
12. Mohammed El-Kaseem, et al, Assay and dissolution methods development and validation for simultaneous determination of sofosbuvir and ledipasvir by RP-HPLC method. Juniper publishers2017, 1(3), 0001-0011.
13. Battulasreenivasarao, et al, Simultaneous analysis of ledipasvir and sofosbuvir in bulk and tablet dosage form by stability indicating high performance liquid chromatographic method. Gjra2017, 6(4), 505-509.
14. Ravi kumar, et al, Estimation and validation of sofosbuvir in bulk and tablet dosage form by RP-HPLC. Int J pharm2016, 6(2), 121-127.
15. Suryaprakash, et al, Development and validation of RP-HPLC and UV spectroscopy methods for simultaneous estimation of sofosbuvir and ledipasvir in their combined tablet dosage form. Pharma Science Monitor2017, 8(2), 369-388.