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Simultaneous Estimation of Sofosbuvir and Ledipasvir in Human Plasma in Bulk Form by RP-HPLC

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Abstract

This study's goal is to assess Sofosbuvir and Ledipasvir in human plasma using the RP-HPLC technology. The separation was performed using a Hypersil (ODS) C18 (4.6 x 250mm, 5 m) analytical column. The mobile phase was a 60:40 mixture of methanol and water. With the help of a UV detector set at 270.0 nm, the eluents were found. The discovered approach produced Sofosbuvir and Ledipasvir retention periods that were determined to be 2.1 and 5.6 min, respectively. ICH guidelines were followed to verify the procedure with regard to precision, specificity, accuracy, linearity, and stability tests. It was discovered that the suggested procedure was practical and reproducible for quantitative evaluation of Sofosbuvir and Ledipasvir.

Keywords: ICH, HPLC, Sofosbuvir, Ledipasvir.

Full length article **Corresponding Author*, e-mail: pavanich.pharma@gmail.com,

1. Introduction

This study set out to develop and validate a simple method for measuring the most commonly used drugs for treating hepatitis C virus (HCV) infection—Human plasma containing sofosbuvir and ledipasvir—in accordance with ICH guidelines.[1, 2] Sofosbuvir is a medication. It is transformed into the potent antiviral compound GS-461203. The viral RNA polymerase, the NS5B protein, uses GS-461203 as a faulty substrate, which inhibits the synthesis of viral RNA. [3, 4, 5, 6, 7] Hepatitis C virus infection can also be treated with ledipasvir. Ledipasvir blocks NS5A, a crucial viral phosphoprotein essential in viral replication assembly and secretion. The FDA authorized the use of sofosbuvir (SBR) and ledipasvir (LDR) together in 2014 to treat HCV. [8, 9, 10, 11, 12, 13, 14, 15, 16] A reliable, straightforward, and verified technique for simultaneously quantifying sofosbuvir and ledipasvir in human plasma must be developed and reported. So, in order to design a quick, simple, and reliable HPLC technique for the drugs sofosbuvir and ledipasvir, as well as to verify it in accordance with the ICH Q2B guidelines for the development and validation of analytical methods, we made an effort to eliminate the shortcomings in the prior methods.

2. Materials and Methods

Chemicals:

Hetero Labs kindly provided pharmaceutical-grade Sofosbuvir and Sofosbuvir as a chemical contribution. For this work, analytical reagent grade solvents and chemicals were given by FINER Chemical LTD, Sigma Aldrich (Mumbai), and Lichrosolv (Merck)

Instruments:

Symmetry ODS C18 (4.6 x 250mm, 5 m) column used for separation; Lab solutions software was used for monitoring and integrating the output signal; spectrophotometer was a Systronics PC-based 2202 with matching quartz cells, 1 cm.

HPLC method development:

Mobile phase preparation:

A – Methanol
B – pH-balanced (with 0.05% acetic acid) water
Degas A and B separately for 5 minutes in an ultrasonic water bath. Filter using a 0.45 filter in a hoover. Then, combine components A and B in an 83:17 ratio.
Diluent Preparation: The Mobile phase served as the diluent.

Wavelength Selection:
Ledipasvir and Sofosbuvir sample and standard solution preparation:
Standard Solution preparation:

The stock mixture of sofosbuvir and ledipasvir were made by dissolving the proper quantity into the diluent at concentrations of 1 mg/ml. At 2 to 80C, all of the stock solutions were kept. Sofosbuvir and ledipasvir stock solutions were further diluted with diluent to create standard mixes, yielding final concentrations ranging from 1000ng/mL to 5000ng/mL. Ledipasvir and Sofosbuvir were produced in a typical 1:1 combination with 1000ng/mL each in the diluent.

Preparation of sample Solution:

Sofosbuvir and Ledipasvir were extracted from plasma samples using an easy two step liquid-liquid extraction (LLE) process. 200µL of plasma and 500µL of previously prepared medication solutions were combined with acetonitrile for deprotination, and 20 minutes were spent centrifuging the mixture at 5000 rpm and 40 degrees Celsius. The needed amount of the organic layer was removed, diluted with methanol to 10 ml, and then this solution was added to the HPLC apparatus.

Procedure:

The assay's % must be calculated by first injecting 10 □L of sample and standard and into the chromatographic apparatus, measuring the sofosbuvir and ledipasvir peak regions, and then using the formulae.

Optimization of Colum:

At a flow rate of 1.0 ml/min, the best results were achieved using a (ODS) C18 column (4.6 x 250mm, 5 m, Make: Hypersil).

RESULTS:
Method validation:
System suitability study:

Sofosbuvir and Ledipasvir were tested at a concentration of 2000ng/ml in six independent assays to determine the system's efficacy. The % relative standard was computed taking into consideration the theoretical plate, retention period, and asymmetry factor.

- The average Tailing factor for Sofosbuvir and Ledipasvir were found to be 1.197, 1.028 respectively.
- There must be at least 2000 plates in theory.
- Peak tailing should be no more than 2

Specificity:

Analyzing blank and reference samples can help identify the selectivity. Selectivity was confirmed at a lower limit of quantification (LLOQ) after analysis of interference in a blank sample

Linearity:

For all procedures, a single 5-point calibration curve was created. With the help of linear regression and the least squares approach, the findings were utilized to derive the equation of the line.

Procedure for calibration curve:

The injected Sofosbuvir and Ledipasvir concentrations ranged from 1000 to 5000ng/mL, and the extracted plasma samples were then used to produce the appropriate chromatograms. These chromatograms were used to compute each dilution's area under the drug's retention times and curve relative to the reference standard. The concentration and area under the calibration curve were plotted on the x- and y-axes, respectively, to create a useful calibration curve. 1000–5000ng/ml was discovered to be the linearity range. Calculated was the curve's regression equation.

Acceptance Criteria:

Each drug's concentration versus peak area should be plotted linearly with an R2 correlation coefficient that is no higher than 0.999.

Accuracy:

The accuracy of an analytical procedure is measured by how closely the results of the analysis match the true value. This was determined by conducting recovery trials with known concentrations of standard S and L (50%, 100%, and 150%) as part of the analytical technique. Percentages of success after treatment were derived from this data.

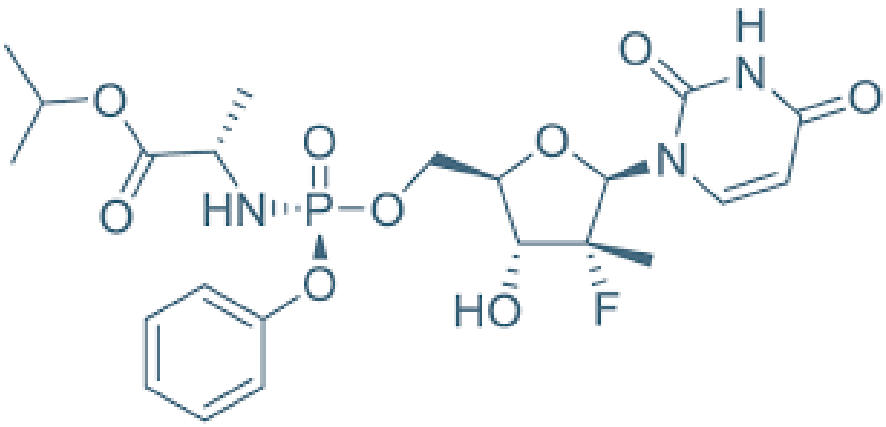

Fig. 1: Sofosbuvir's Molecular Structure

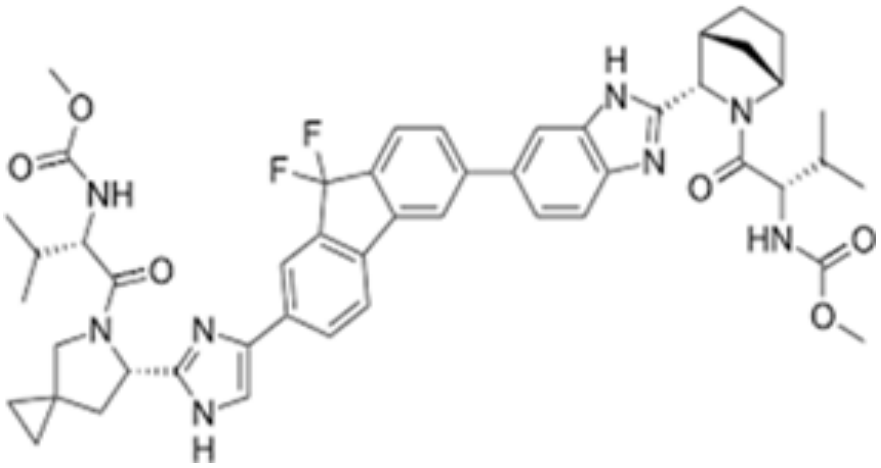

Fig. 2: Ledipasvir's Molecular Structure

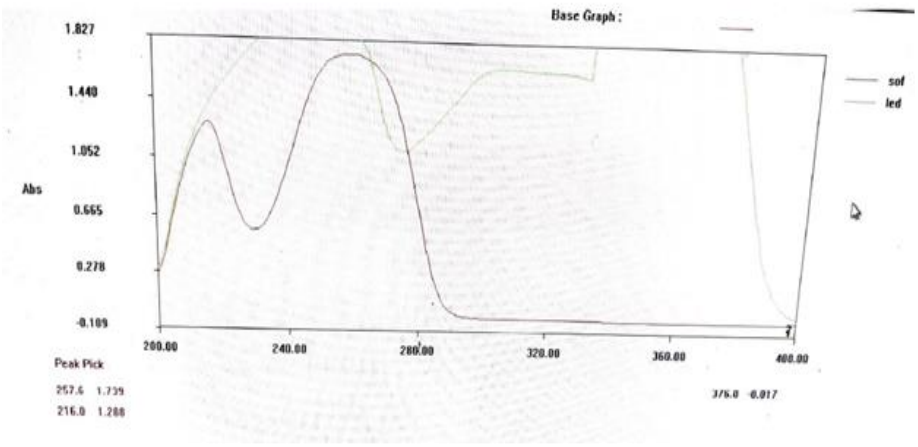

Fig. 3: UV-Spectrum of Ledipasvir and Sofosbuvir

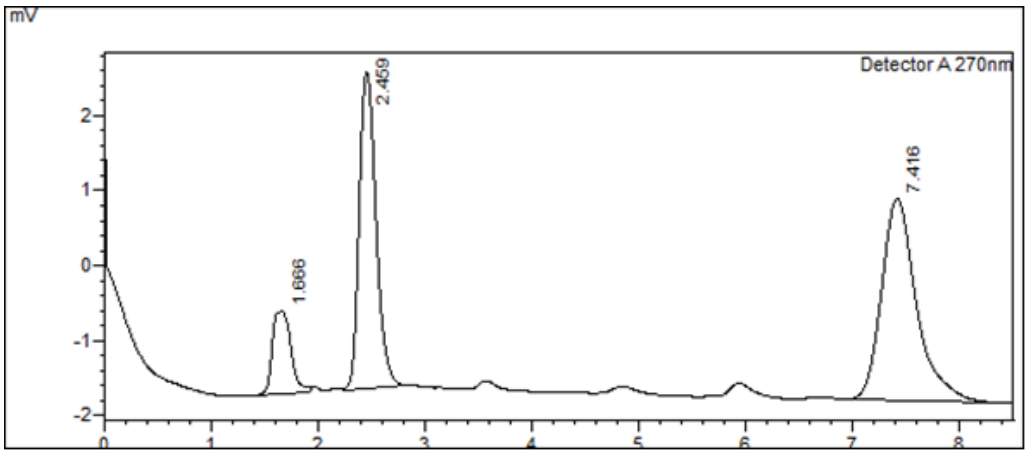

Fig. 4: Optimized Chromatogram

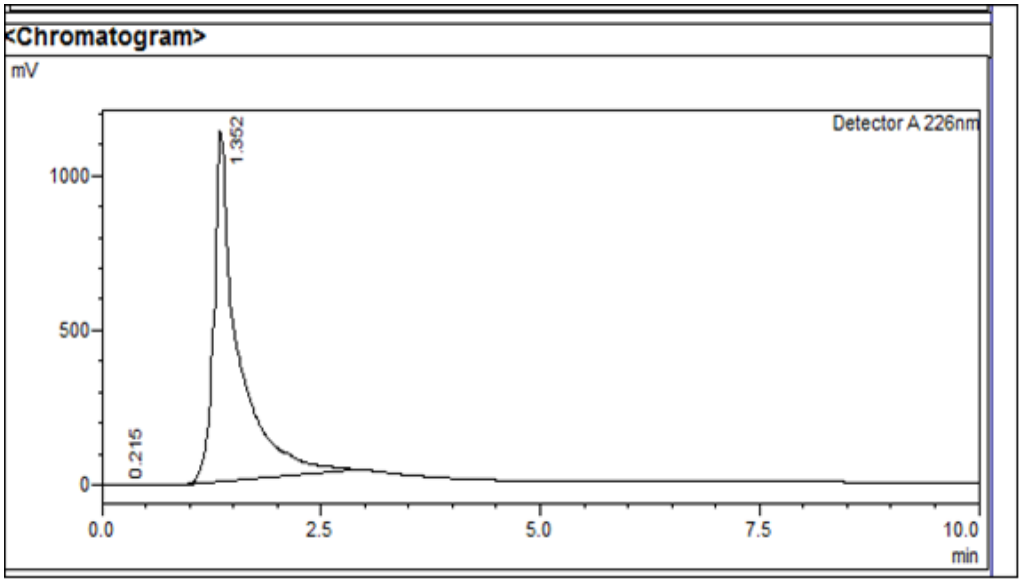

Fig. 5: Plasma Chromatogram

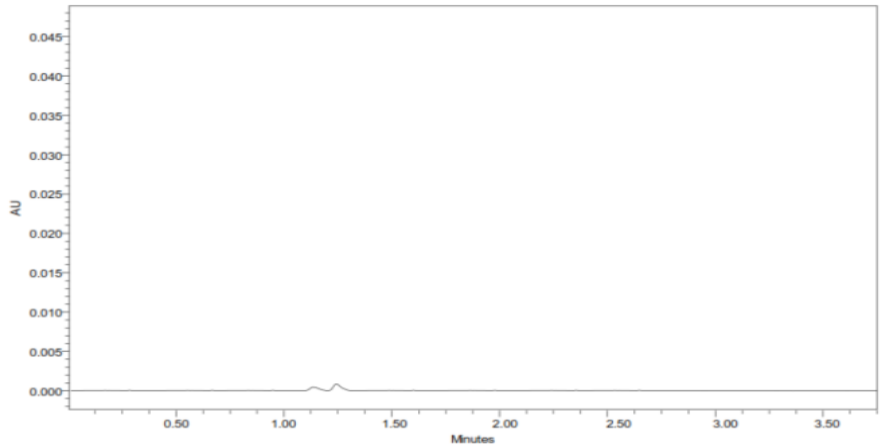

Fig. 6: Represents Blank Chromatogram

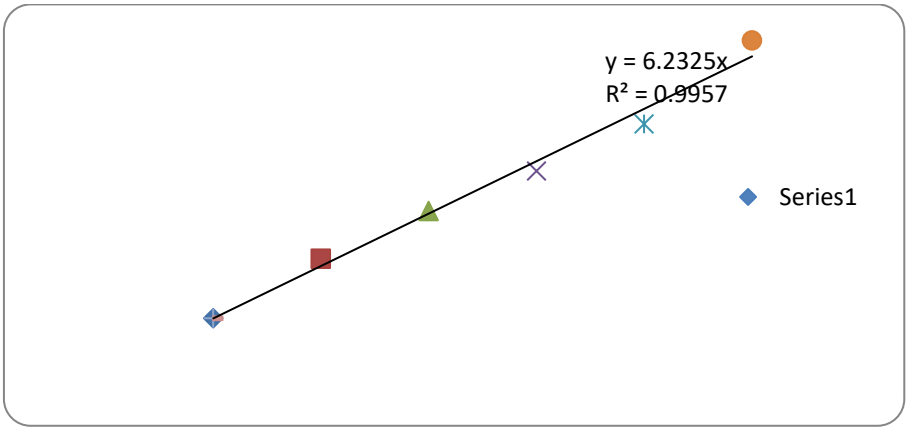

Fig. 7: Calibration curve for Sofosbuvir

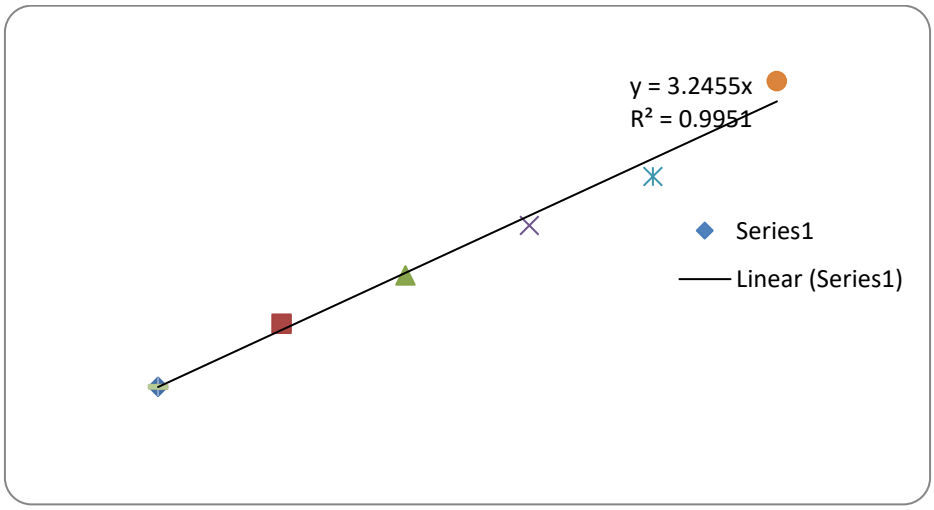

Fig. 8: Ledipasvir Calibration curve

Table 1: Optimized chromatographic conditions

PARAMETERS	CONDITIONS
Column(Stationary Phase)	(ODS) C18 (4.6 x 250mm, 5um,Make:Hypersil)
Mobile Phase	pH-balanced methanol and water (83:17) with 0.05% acetic acid
Flow rate (ml/min)	1
Run time (min)	10
Column temperature(°C)	Ambient
Injection loop volume (µl)	20
Detection wavelength (nm)	270
Drug Retention time (min)	Sofosbuvir -2.113,Ledipasvir-5.619
Resolution	3.291 9.591
USP Plate count	Sofosbuvir-6717 ,Ledipasvir-15576
USP Tailing	Sofosbuvir-1.197,Ledipasvir-1.028

Table 2: System suitability Parameters

Sample Name	Peak area		Retention time		No.of theoretical plates	
	Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir	Sofosbuvir	Ledipasvir
Injection1	12747	6334	2.459	7.713	6717	15576
Injection2	12826	6342	2.139	7.695	6810	15679
Injection3	12506	6443	2.172	7.351	6721	15726
Injection4	12341	6434	2.139	7.695	6694	15567
Injection5	12221	6376	2.172	7.351	6726	15570
Injection6	12332	6332	2.328	7.793	6754	15594
%RSD	1.9	0.7	1.5	0.14	Avg:6737	Avg:15618

Table 3: Sofosbuvir Linearity results

S.No	Linearity Level	Concentration (ng/mL)	Area
1	I (Lower limit of Quantification)	1000	7078
2	II	2000	12747
3	III(Middle Quality Control)	3000	17513
4	IV	4000	23091
5	V(Higher Quality Control)	5000	33062
Coefficient of Correlation			0.986

Table 4: Linearity results of Ledipasvir

S.No	Linearity Level	Concentration (ng/mL)	Area
1	I (Lower limit of Quantification)	1000	3584
2	II	2000	6335
3	III(Middle Quality Control)	3000	9169
4	IV	4000	11963
5	V(Higher Quality Control)	5000	17383
Coefficient of Correlation			0.986

Table 5: Sofosbuvir **Accuracy results**

Sample ID	Concentration (ng/mL)		Response	%Recovery	Statistical Analysis
	Amount added (ng)	Amount found (ng)			
LQC	1000	986.6	6984	98.6	**Mean** = 98.42
LQC	1000	989.8	7005	98.98	
LQC	1000	977.4	6915	97.7	
MQC	3000	3001	17514	100	**Mean** = 96.4
MQC	3000	2858.16	16672	95.2	
MQC	3000	2821.20	16462	94.0	
HQC	5000	5000	33062	100	**Mean** = 102.11
HQC	5000	5191	34318	103.8	
HQC	5000	5127.4	33901	102.54	98.97%

Table 6: Ledipasvir Accuracy results

Sample ID	Concentration (ng/mL)		Response	%Recovery	Statistical Analysis
	Amount added (ng/ml)	Amount found (ng/ml)			
LQC	1000	98.2	3543	98.9	**Mean** = 97.16
LQC	1000	969.43	3473	96.94	
LQC	1000	956.43	3426	95.64	
3.5 MQC	3000	2943.6	8984	98.08	**Mean** = 97.73
MQC	3000	2984.6	9114	99.4	
MQC	3000	2872.4	8775	95.73	
HQC	5000	5000	17381	100	**Mean** = 99.11
HQC	5000	4897.78	17015	97.9	
HQC	5000	4972.4	17283	99.44	98.03%

Table 7: Accuracy data for Sofosbuvir at LLOQ level

Sample ID		Concentration (ng/mL)		Response	%Recovery	Statistical Analysis
		Amount added (ng)	Amount found (ng)			
50 %	1	500	482.5	5058	103	**Mean** = 100
	2	500	513.5	4996	102.7	
	3	500	476.5	4628	94.5	
100 %	1	1000	966.5	68355	96.65	**Mean** = 97.41
	2	1000	980.	6991	98.096	
	3	1000	975.5	6904	97.5	
150 %	1	1500	1460	9589	97.3	**Mean** = 97.6
	2	1500	1480	9718	98.6	
	3	1500	1456	9560	97	**Avg =98.33**

Table 8: Accuracy data for Ledipasvir at LLOQ level

Sample ID		Concentration (ng/mL)		Response	%Recovery	Statistical Analysis
		Amount added (ng)	Amount found (ng)			
50 %	**1**	500	487	1775	97.4	**Mean** = 98.83
	2	500	508	1841	101	
	3	500	489	1768	97.8	
100 %	**1**	1000	970.2	3486	97.02	**Mean** = 97.87
	2	1000	980.6	3532	98.6	
	3	1000	980	3512	98	
150 %	**1**	1500	1440	5250	.96	**Mean** = 97.4
	2	1500	1480	5392	98.6	
	3	1500	1464	5337	97.6	
						98.03 %

Table 9: Sofosbuvir recovery data

Sample ID	Concentration (ng/mL)		Response	%Recovery	Statistical Analysis
	Amount added (ng/mL)	Amount found (ng/mL)			
LQC	1000	897.43	6451	89.74	**Mean** = 91.70%
MQC	3000	2762.4	16126	92.08	
HQC	5000	4686.4	308456	93.3	

Table 10: Recovery data for Ledipasvir

Sample ID	Concentration (ng/mL)		Response	%Recovery	Statistical Analysis
	Amount added (ng)	Amount found (ng)			
LQC	1000	864.18	3096	86.418	**Mean** = 89.26%
MQC	3000	2820	8616	94	
HQC	5000	4569	15192	87.41	

Table 11: Intra-day Precision results for Sofosbuvir and Ledipasvir

Injection	Peak area	
	Sofosbuvir	Ledipasvir
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	465.88	94.50
%RSD	0.39	1.02

Table 12: Inter-day Precision results for Sofosbuvir and Ledipasvir

Injection	Peak area	
	Sofosbuvir	**Ledipasvir**
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	352	71.15
%RSD	1.97	0.76

Table 13: Precision data at LLOQ for Sofosbuvir and Ledipasvir

Injection	Peak area	
	Sofosbuvir	**Ledipasvir**
Injection-1	7079	3584
Injection-2	6922	3549
Injection-3	7095	3674
Injection-4	7057	3524
Injection-5	6962	3583
Injection-6	6957	3528
Average	7013	3572.84
Standard Deviation	178.35	58.33
%RSD	1.04	1.6

Table 14: Freeze and Thaw Stability data

Name of the drug	Amount added	Amount found (ng)	Response	% change
Sofosbuvir	LQC(1000 ng/ml)	986	6978	1.4
		974	6894	2.6
		984	6964	1.6
	MQC (3000 ng/ml)	2895	16900	3.5
		2942	17174	1.9
		2924	17069	2.52
Ledipasvir	LQC (1000 ng/ml)	972.5	3484	2.75
		986.5	3534	1.35
		973	3486	2.7
	MQC (3000 ng)	2923	8931	2.56
		2913	8901	2.9
		2906	8879	3.13

Table 15: Short-term temperature stability data

Name of the drug	Amount added	Amount found	Response	% change
Sofosbuvir	LQC (1000ng)	966.6	6841	0.625
		974.8	6899	2.52
		976.4	6910	2.36
	MQC (3000 ng)	2924	17069	1.52
		2899	16923	2.02
		2989	17448	0.22
Ledipasvir	LQC (1000ng)	989.9	3546	1.01
		976.4	3498	2.36
		986.4	3534	1.36
	MQC (3000 ng)	2912	8898	1.76
		2811.5	8591	3.78
		2809.4	8584	3.812

Table 16: Long-term temperature stability data

Name of the drug	Amount added	Amount found (ng)	Response	% change
Sofosbuvir	LQC (1000 ng/ml)	984.9	6971	1.51
		965.4	6833	3.46
		985	6971	1.5
	MQC (3000 ng/ml)	2942	14232	2.5
		2895	16900	3.5
		2924	17069	1.52
Ledipasvir	LQC (1000 ng/ml)	992	3554	0.8
		978	3504	2.2
		986	3532	1.4
	MQC (3000 ng/ml)	2911	8895	2.96
		2963	9053	1.2
		2972	9081	0.93

Table 17: Stock Solution Stability data

Name of the drug	Amount added	Amount found	Response	% change
Sofosbuvir	1000 µg	996 µg	71426	0.5 %
Ledipasvir	1000 µg	998 µg	35722	0.79 %

Acceptance criteria:
Nominal concentrations should be within 15% of stability sample findings.

Acceptance limit:

The average recovery should range from 98 to 102%.

Recovery:

To determine recovery, the drug concentrations in the aqueous solution and the spiking solution were compared. The levels of recovery were determined by comparing the analytical results from three different concentrations of extracted samples (LQC, MQC, and HQC) to those from corresponding standards that had not been extracted. The results are summarised in Tables 10 and 11.

Precision and Intermediate precision:

When the process is performed on several aliquots of a single homogeneous biological matrix volume, analytical precision is helpful for characterizing the closeness of individual analyte readings.. Tables 6 and 7 show the results in tabular form.

Stability Studies:

i. Freeze and Thaw Stability:

Three rounds of freezing and thawing verified the sample's stability. Three different samples of LQC and MQC were frozen for 24 hours before being allowed to defrost at room temperature. Once the samples were at room temperature again, they were frozen again for another 12-24 hours. The freeze-thaw cycle was carried out three times in total, the third being used for analysis.

ii. Short-Term Temperature Stability:

After allowing three aliquots of LQC and three aliquots of HQC to warm to room temperature for 22 hours, they were analyzed.

iii. Long-Term Stability:

Long-term stability assessments need a longer period of storage than is required to collect the first sample and complete the final analysis. Thus, in order to assess their long-term stability, three aliquots of LQC and HQC were kept in the same conditions as the research materials for a total of 22 days.

Stock Solution Stability:

It took six hours to assess the stability of medication stock solutions at room temperature. Results from a stability sample must fall within 15% of nominal concentrations.

DISCUSSION:

Sofosbuvir and Ledipasvir were quantified in human plasma using a straightforward bioanalytical approach. In order to create the bio analytical HPLC method, we employed a (ODS) C18 (4.6 x 250mm, 5um, Make: Hypersil) column with a runtime of 10 minutes. The protein precipitation method was employed to get samples ready for analysis. The mobile phase used in this study was composed of acetonitrile, methanol, and water at a ratio of 60:20:20, and the flow rate was set at 1 mL/min. The procedure's mobile phase was simple and inexpensive to set up. The range of the percentage mean recovery was determined to be 89.7-93.3% for sofosbuvir and 86.80-92.5% for ledipasvir. For sofosbuvir and ledipasvir, the number of theoretical plates is more than 2000, and the tailing factor is less than 2.0. The accuracy of the system and procedure was evaluated, and it was found to be within acceptable ranges. Herein is laid down the precise methodology. Studies assessing the procedure's precision found a recovery value of 99.97% to 100.04% for pure medication and sample.The developed system met all of those criteria, plus it was fast, accurate, and easy to use. It was found that Ledipasvir and Sofosbuvir were stable under a range of stability settings. Comparing the recommended extraction process to previously published methods; it is noticeably more straightforward, quick, reliable, and sensitive. This approach is better suited to processing several samples quickly for pharmacokinetic research because to its straightforward sample preparation process and fast chromatographic duration. This technique satisfied the ICH-established requirements for validation. As a result, the devised approach may be used for human therapeutic medication monitoring and pharmacokinetic investigations.

4. Conclusions

The empirical evidence suggests that Sofosbuvir and Ledipasvir can be identified simultaneously by RP-HPLC. The new method was found to be superior to the old ones in every respect. All of the APIs were determined to be relevant and resolute under conditions ideal for simultaneous evaluation in bulk form and permitted dosage form.

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] B.K. Sharma. "Instrumental Methods of Chemical Analysis, Introduction to Analytical Chemistry," 23rd ed. Goel Publishing House, Meerut, 2004, pp. 12-23.
[4] P.D. Sethi. "HPLC: Quantitative Analysis of Pharmaceutical Formulations," CBS Publishers and Distributors, New Delhi (India), 2001; pp. 3-137.
[5] [Wikipedia] Sofosbuvir. (URL: https://en.wikipedia.org/wiki/Sofosbuvir)
[6] [Wikipedia] Hepatitis C. (URL: https://en.wikipedia.org/wiki/Hepatitis_C)
[7] [Wikipedia] Ledipasvir. (URL: https://en.wikipedia.org/wiki/Ledipasvir)
[8] B. Zaman et al., "RP-HPLC Method for Simultaneous Determination of Sofosbuvir and Ledipasvir in Tablet Dosage Form: Its Applications to In vitro Dissolution Studies," Springer Link, Chromatographia, 2016; 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 Sciences, 2016; 5(8), 1312-1321.

[10] K.K. Kranthi et al., "A New Analytical Method Development and Validation for the Simultaneous Estimation of Ledipasvir and Sofosbuvir Using RP-HPLC," ICJPIR, 2017; 4(1), 142-165.

[11] Yogendrachari et al., "Analytical Method Development and Validation for Simultaneous

[12] Determination of Ledipasvir and Sofosbuvir in Tablet Dosage Form by RP-HPLC," J Global Trends Pharm Sci, 2016; 7(3), 3386-3393.

[13] B. Ramu, Kaushal K. Chandrul, P. Shanmuga Pandiyan. "Using 24 Factorial Designs Optimization of Repaglinide Gastroretentive Drug Delivery System," Research J. Pharm. and Tech., 2021; 14(2), 725-729.

[14] B. Sreenivasa Rao et al., "Simultaneous Analysis of Ledipasvir and Sofosbuvir in Bulk and Tablet Dosage Form by Stability Indicating High-Performance Liquid Chromatographic Method," GJRA, 2017; 6(4), 505-509.

[15] Ravi Kumar et al., "Estimation and Validation of Sofosbuvir in Bulk and Tablet Dosage Form by RP-HPLC," Int J Pharm., 2016; 6(2), 121-127.

[16] 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 Monitor, 2017; 8(2), 369-388.

[17] C. Pavani and V. Jayashree. "Development of a Novel Stability Quoting RP-Ultra Performance Liquid Chromatography Approach for Synchronous Assessment of Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate in Pure API Form and Tablet Dosage Based on ICH Guidelines," J. Drug Alcohol Res., 2021; 11, 1-7.