METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF SOFOSBUVIR AND LEDIPASVIR IN BULK AND TABLET DOSAGE FORM BY RP-HPLC

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Running title : method validation of bulk and tablet dosage form by RP- HPLC method for Sofosbuvir and Ledipasvir.

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ABSTRACT

The goal of the current study was to develop and validate an RP- HPLC method which is simple, specific, precise and accurate for the estimation of Sofosbuvir and Ledipasvir in bulk and pharmaceutical dosage forms. The chromatographic separation was accomplished on a Phenomenex Luna C_{18} column with 4.6 x 250mm internal diameter and 5µ particle size packing. 20mM Phospate buffer (pH 3.0): Methanol in the ratio of 15:85% v/v was used as a mobile phase and an isocratic method of elution was applied. Standard and sample was detected using UV detector set at 260.0nm and LC solution software was used for data analysis. The developed method was validated according to ICH guidelines. The linearity for Sofosbuvir in concentration range of 5-25 µg/ml and Ledipasvir was 1.125-5.625 µg/ml considered good. The retention time of Ledipasvir was 4.04 minutes and Sofosbuvir was 2.05 minutes. LOD and LOQ values for Sofosbuvir and Ledipasvir were determined to be 0.69 & 3.12 and 0.012 & 3.66 respectively. Relative standard deviation obtained for Sofosbuvir was 1.23 and Ledipasvir was 0.84. Hence this method can be used for analyzing huge number of samples in less time due to shorter retention times. It is concluded that the developed method can be effectively applied for routine analysis of Sofosbuvir and Ledipasvir in bulk and pharmaceutical dosage forms.

Keywords: Sofosbuvir, Ledipasvir, Method validation, RP- HPLC, Isocratic elution.

1. INTRODUCTION

Sofosbuvir and Ledipasvir are used to treat hepatitis C which is the primary cause of liver cancer. Ledipasvir is used in combination with other medications to treat genotype 1 hepatitis. Hepatitis C NS5A and NS5B proteins are blocked or inhibited by Ledipasvir and Sofosbuvir respectively[1].

Drug transporters P-gp substrates include Ledipasvir and Sofosbuvir. 95% of patients are cured by this antiviral medication, which also lowers the death rate.

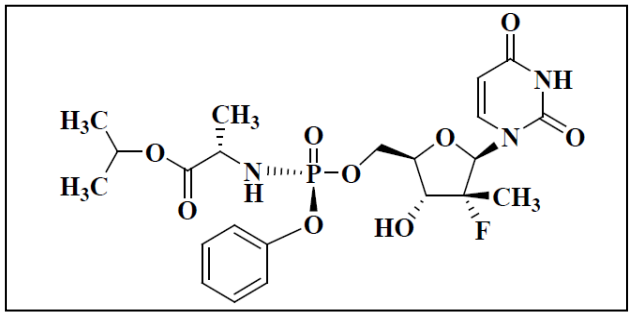

Figure 1 : Chemical structure of Sofosbuvir

The chemical name of Sofosbuvir is Isopropyl (2S)-2-[[(2R,3R,4R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methylfuran-2-yl]methoxy-phenoxyphosphoryl]amino] propionate. It have a molecular formula of C22H29FN3O9P.

Sofosbuvir alone used for the treatment of **COVID-19**, due to similarity between the replication mechanism of HCV and Corona virus. Covid-19 is an infectious disease caused by corona virus. It may lead to death when individual having low immune power[2-4].

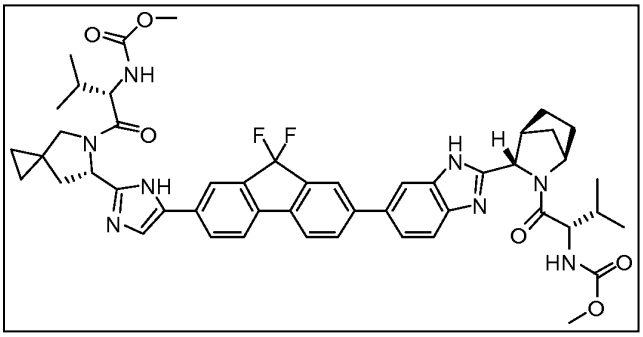

Figure 2 : Chemical structure of Ledipasvir

The chemical name is (2S)-1-[(6S)-6-[5-(9,9-difluoro-7-(2-[(1R,3S)-2-(2S)([hydroxyl (methyl) methylidene amino)-methylbutanoyl]-2 azabicyclo [2,2,1]heptan-3-yl-1H-1,3- benzodiazol-6-yl)-9H-fluoren-2-yl)-1H-imidazole-2-yl]-5-azaspiro[2.4]heptan-5-yl]-2-([hydroxyl(methoxy)methylidene] amino)-3- methylbutan-1-one. it has a molecular formula of C49H54F2N8O6.

Ledipasvir and Sofosbuvir [90/400 mg] tablet dosage form is approved by **CDSCO (08.12.2015)**[5]. GILEAD Sciences Inc, Ireland (2014), discovered the fixed dose of combinational drug which was traded as HARVONI. Following are the list of research andreview done in Sofosbuvir and Ledipasvir tablet dosage form.

Literature survey reveals that very less methods or studies are done on this combination of Sofosbuvir and Ledipasvir[6-13]. The main objectives of this current study was to develop a simple precise, accurate, and economical method with reproducible assay results for combined tablet dosage form of Ledipasvir and Sofosbuvir and it was validated according to ICH Q2 (R1) Guidelines[14].

High Performance Liquid Chromatography is a technique used for separation of various compounds such as high molecular weight, less stable and even ionic species. HPLC can be used for both qualitative and quantitative analysis. It works on the basis of absorption, ion exchange, partition and size exclusion. The wide choice of stationary phase makes it more desirable. It is also useful in monitoring the contaminants present in the bulk or even the formulations. It also facilitates the use of

polar and non polar solvents as well. It is a non- destructive method, applied for wide range of compounds. The developed method should be time saving as it is a notable criteria in drug development. It aids in structure clarification and quantitative conviction of impurities and deterioration products in bulk drug materials and pharmaceuticals constitutions [15].

2. MATERIALS AND METHODS

2.1. Chemicals

The pure Active pharmaceutical ingredient i.e., Sofosbuvir and Ledipasvir were received as gift sample from HETERO LABS LIMITED (H.P, INDIA). MyHep LVIR [400/90 mg], the commercial formulation in tablet form was procured from medical store.

2.2. Instrumentation

The HPLC instrument included Shimadzu (LC 20 AD). Separation and quantitation was done by using Phenomenex Luna C18 column with 4.6x250 mm id and 5µm particle size packing. The HPLC system is equipped with various sensitive detector mainly UV detector (PDA detector) which is used for the detection of components and LC solution software for data acquisition. UV Spectroscopy (Shimadzu 1800) was used to determine the wavelength of the Ledipasvir and Sofosbuvir. Each compound is measured separately many times with various solvents. The λmax for Sofosbuvir and Ledipasvir was found to be 210 and 237 nm respectively. The isobestic point was determined by using overlay spectra for simultaneous drug estimation, and it was found to be 260 nm.

2.3. Chromatographic conditions

The choice of method is dependent on the nature, molecular weight and solubility of the sample. Ledipasvir and sofosbuvir are freely soluble in organic solvents. Hence, RP - HPLC opted the mobile phase which is polar. The separation of both Active pharmaceutical ingredient and drug formulation carried out by Shimadzu (LC 20 AD) module with PDA detector for detection. The analytical column Phenomenex Luna C18 column with 4.6x250 mm id and 5µm particle size and use of mobile phase containing mixture of 20 mM Phosphate buffer (pH was adjusted to 3.0 by using orthophosphoric acid):Methanol were taken at ratio of 15: 85% v/v, which express a good symmetrical peak with efficient separation of drugs. Ambient temperature was maintained. The eluents were monitored at 260nm with a flow rate of 1.5 ml/minute.

2.4. Preparation of Mobile phase

A mobile phase is chosen based on the solubility of the sample. Ledipasvir and Sofosbuvir are soluble in HPLC grade water and methanol on ultrasonication. It is completely insoluble in hexane and freely soluble in DMSO. Methanol and 20 mM Phosphate buffer were selected as mobile phase for this method development and it was prepared by mixing 20 mM Phosphate buffer and Methanol in the ratio of 15:85% v/v and pH was adjusted to 3.0 with orthophosphoric acid. Prepared buffer solution was filtered by using 0.45µ membrane filter. The mobile phase was sonicated for 20 minutes and the same was used as diluents for further dilution.

2.5. Preparation of standard solution

Standard solutions of drug were prepared by accurately weighing 400 mg of Sofosbuvir and 90 mg of Ledipasvir and transferred it into 100 ml volumetric flask and 100ml of diluent were added (Phosphate buffer: Methanol 15: 85% v/v). From the stock solution 10 ml was taken and transferred into 100 ml volumetric flask and diluted with diluent up to the makeup volume. To achieve the desired concentration, the stock solution, which included 400 µg/ml of Sofosbuvir and 90 µg/ml of Ledipasvir, was further diluted.

2.6. Preparation of test solution

The test solutions of drug formulation were prepared by accurately weighing 20 tablets and crushed to powder. Then transfer weight equivalent to 400 mg of Sofosbuvir and 90 mg of Ledipasvir into100 ml volumetric flask and 100ml of diluent were added (Methanol: Phosphate buffer 85:15). 10 ml of the above stock solution was taken in 100 ml volumetric flask and diluted with diluent up to the

mark. To achieve the desired concentration, the stock solution, which included 400 µg/ml of Sofosbuvir and 90 µg/ml of Ledipasvir, was further diluted.

2.7. Optimization of method

Detection of wavelength of the standard solution of the drugs was obtained from the PDA detector. The wavelength range for both medicines was 190 to 400 nm. The isobestic wavelength after scanning overlain spectra was 260 nm.

3. RESULTS

3.1. Method Validation

The method validation was performed as per ICH Q2 (R1) guidelines, it was determined bye examining parameters such as specificity, accuracy, precision , linearity, robustness, (LOD), and (LOQ), system suitability.

3.2. Optimizetion of Chromatographic conditions

Mobile phase containing mixture of 20 mM Phosphate buffer (pH was adjusted to 3.0 by using orthophosphoric acid): Methanol were taken at ratio of 15: 85% v/v, which express a good symmetrical peak with efficient separation of drugs. Ambient temperature was maintained. The eluent was detected at 260nm and the flow rate was maintained at 1.5 ml/minute. The retention time was observed at 2.05 minutes for Sofosbuvir and 4.04 minutes for Ledipasvir respectively.

Optimized of Chromatographic conditions

Elution type : Isocratic elution
Stationary Phase : Phenomenex Luna C_{18} column
Mobile Phase : 20 mM Phosphate buffer (pH- 3.0) , Solvent B – Methanol
Solvent ratio : 15 : 85 % v/v (A : B)
Wavelength for detection: 260 nm
Flow rate : 1.5 ml/min
Sample volume : 20 µl
Temperature : Ambient
Retention time : 2.05 min for Sofosbuvir and 4.04 min for Ledipasvir respectively.

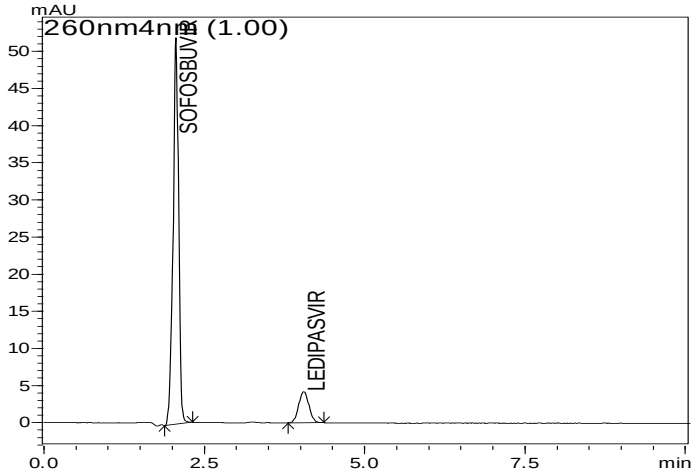

Figure 3: Optimized Chromatogram for Sofosbuvir and Ledipasvir

3.3. Linearity

The linearity of Sofosbuvir was found to be in the concentration range of 5-25 µg/ml and Ledipasvir was found to be in the concentration range of 1.125 – 5.625 µg/ml respectively. Both the drugs found to be linear and correlation co-efficient were found to be 0.9987 and 0.9979 respectively. The calibration curves were plotted as peak area Vs concentration of the standard solutions (figure 4 and 5).

Table 1: Linearity of Sofosbuvir and Ledipasvir

S.No	Conc of Sofosbuvir (µg/ml)	Peak area of Sofosbuvir	Conc of Ledipasvir (µg/ml)	Peak area of Ledipasvir
1.	5	100679	1.125	13921
2.	10	188626	2.25	26857
3.	15	276027	3.375	38789
4.	20	368176	4.5	51566
5.	25	456535	5.625	65418

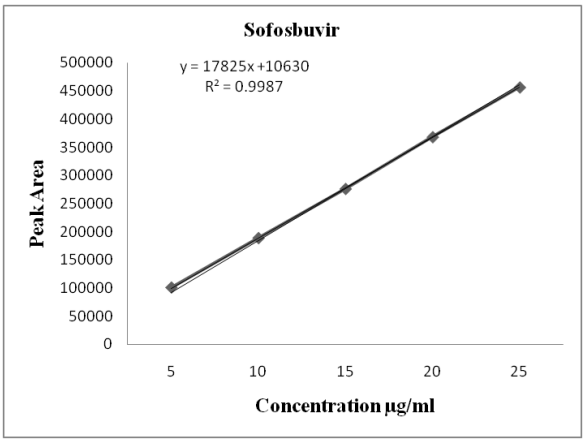

Figure 4: Calibration curve of Sofosbuvir

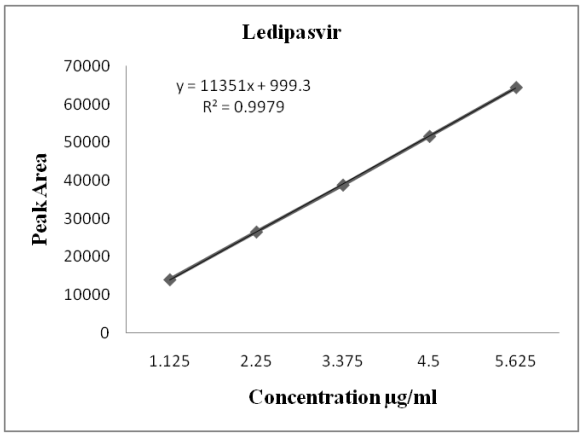

Figure 5: Calibration curve of Ledipasvir

3.4. Accuracy

The standard addition approach was used for accuracy studies. Recovery experiments were used to determine accuracy. A well-known amount or concentration of the pure drug was injected and It was measured in drug products at 50%, 100%, and 150% levels. The recovery studies were done at 6 times and their percentage relative standard deviation and percentage recovery were calculated and given in Table 2. The percentage recovery of Sofosbuvir and Ledipasvir were found to be in the range of 98.19 to 99.41% and 99.15 to 100.8% respectively.

Table 2: Accuracy of Sofosbuvir and Ledipasvir

Drug	Label claim (mg/tab)	Spike Level (%)	Area	Amount of drug added (µg/ml)	Amount of drug found (µg/ml)	Percentage Recovery*	%RSD*
SOF	400 mg	50	178626	200	196.39	98.19	0.56
		100	263827	400	397.43	99.35	0.23
		150	348176	600	596.46	99.41	0.30
LED	90 mg	50	26920	45	45.37	100.83	0.69
		100	38189	90	89.24	99.15	0.60
		150	52093	135	135.37	100.27	0.13

*Mean of 6 observations

3.5. Precision

The precision of the method was determined by studying reproducibility and repeatability. The area of drug peaks and percentage relative standard deviation of intraday and inter day were calculated and presented in Table 3 and 4. The peak area and retention time of both solutions under study was determined and percentage relative standard deviation was calculated**.**The results obtained complied with acceptance criteria since percentage relative standard deviation of peak areas of Sofosbuvir and Ledipasvir were found to be within the limit i.e. percentage relative standard standard deviation –Not more than 2%.

Table 3: Intraday and Interday Precision of Sofosbuvir and Ledipasvir

Precision	Intraday		Interday	
Parameter	**Peak area of Sofosbuvir**	**Peak area of Ledipasvir**	**Peak area of Sofosbuvir**	**Peak area of Ledipasvir**
Precision	256027	33677	256011	33627
	256082	33684	256062	33634
	256030	33620	256020	33650
	256056	33609	256036	33619
	256094	33614	256054	33684
	256101	33692	256171	33632
Average*	256065	33649.33	256059	33641
%RSD*	0.012	0.115	0.022	0.069

*Mean of 6 observations

3.6. Assay

Table 4: Assay result of Sofosbuvir and Ledipasvir

Drug	Area	% Recovery*	%RSD*
Sofosbuvir	256080	99.10%	1.23
Ledipasvir	38293	99.85%	0.84

*mean of 6 observations

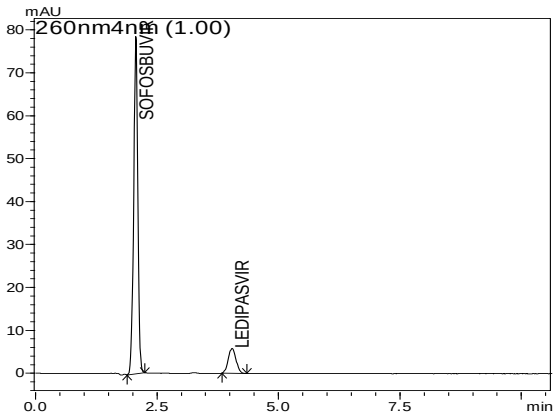

Fig 6: Standard drug chromatogram of Ledipasvir Assay sample

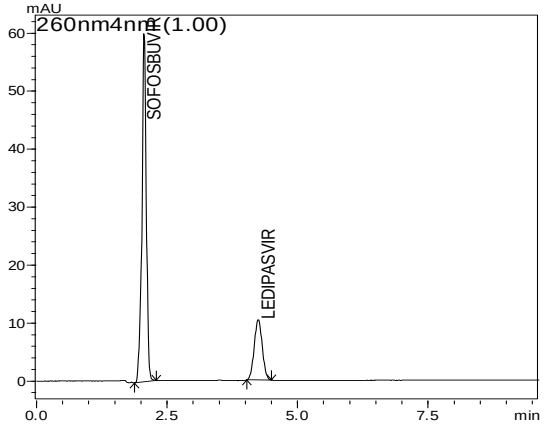

Fig 7: Chromatogram of Sofosbuvir and Sofosbuvir and Ledipasvir

3.7. Limit of Detection and Limit of Quantitation

The LOD and LOQ of Sofosbuvir and Ledipasir were found to be 0.61 & 0.12 µg/ml and 3.12 & 3.66 µg/ml respectively.

Table 5: LOD and LOQ of Sofosbuvir and Ledipasvir

Parameter	Sofosbuvir (µg/ml)	Ledipasvir (µg/ml)
LOD(Limit of Detection)	0.61	0.12
LOQ(Limit of Quantification)	3.12	3.66

3.8. System suitability

The stock solution were prepared by 40mg of Sofosbuvir and 9 mg of Ledipasvir accurately weighed and transferred into 10 ml of volumetric flask and 5 ml of diluent added (20 mM Phosphate Buffer: Methanol in the ratio of 15:85 % v/v) and the particle are completely dissolved by the help of ultraconication, the volume made up to 10 ml by using diluents. This stock solution was further diluted to required concentration. About 20 µl of drug solution was injected under this optimized chromatographic conditions to assess the system suitability, all parameters within the limits according to the ICH guidelines and given in Table 2.

Table 6: System suitability parameter

S. No	Parameter	Sofosbuvir (SOF)	Ledipasvir(LED)
1.	Retention time	2.05	4.04
2.	Tailing factor	1.15	1.08
3.	Peak purity index	0.9996	0.9994
4.	Resolution factor (Rs)	7.31	7.31
5.	No. of theoretical plate (N)	2788.35	3672.76
6.	Height equivalent to theoretical plate (HETP)	88.80	84.15

3.9. Robustness

Table 7: Robustness Data for Sofosbuvir and Laeipasvir

Chromatographic Condition		Sofosbuvir		Ledipasvir	
		Average peak area*	%RSD*	Average peak area*	%RSD*
Mobile phase composition	83:17	377684	0.06	53842.5	0.17
	85:15	348176.6	0.19	51166	0.34
	87:13	341362.4	0.05	50078.3	0.14
pH	2.9	397587.3	0.32	54351.3	0.09
	3.0	348385	0.13	51275.3	0.42
	3.1	321661.5	0.22	49876.5	0.16
Flow rate	1.4 ml	407683.5	0.07	52874	0.08
	1.5 ml	348377.6	0.23	51367	0.13
	1.6 ml	311354	0.16	48678.5	0.22

*Mean of 6 observation

4. DISCUSSION

The Fixed dose combination containing Sofosbuvir (400mg) and Ledipasvir (90mg) is introduced as a direct-acting antiviral and also for Hepatitis C treatment. Literature survey reveals about the several methods used to assess the drug individually and in combination along with other drugs in various pharmaceutical drug dosage form and biological fluids. Very few methods of these two medications in fixed dosage form have been reported. In order to estimate Sofosbuvir and Ledipasvir simultaneously in bulk and tablet dose form, an attempt was made to design a simple, accurate, and precise procedure. Retention time of Sofosbuvir and Ledipasvir were found to be 2.05 minutes and 4.04 minutes. Both the drugs were resolved using 20 mM Phosphate buffer: Methanol in a ratio of 15:85% v/v with a pH adjustment of 3.0. The good resolution was obtained on Phenomenex Luna C_{18} Column (4.6 x 250 mm x 5 µ) column at a maximum wavelength of 260 nm. The correlation co-efficient (R^2) were found to be 0.9987 and 0.9979 respectively. An excellent resolution and the best retention time were achieved between the two medicines. %RSD of the Sofosbuvir and Ledipasvir were and found to be 1.23 and 0.84 respectively. The % Recovery was obtained as 99.10% and 99.85% for Sofosbuvir and Ledipasvir respectively. LOD, LOQ values obtained from regression equations of Sofosbuvir and Ledipasvir were 0.61, 3.12 and 0.01, 3.66 µg respectively.

5. CONCLUSION

The developed method was found to be very sensitive, accurate, precise, simple, robust, and fast. Huge number of samples can be analyzed in less time due to shorter retention times, so the developed method can be effectively applied for routine analysis of Sofosbuvir and Ledipasvir in bulk and pharmaceutical dosage forms.

6. REFERENCES

1. European Commission Final Version of Annex 15 to the EU Guide to Good Manufacturing Practice: Qualification and validation 2001; 4:1-10.
2. G. Lavanya, M. Sunil, M.M. Eswarudu, M. Chinna Eswaraiah, K. Harisudha and B. Naga Spandana, “Chemical Entities of Biological Interest (ChEBI) Sofosbuvir”, International Journal of Pharmaceutical Sciences and Research 2011; 2(3): 20-26.
3. AnahidNourian and Hossein Khalili, “Sofosbuvir as a potential option for the treatment of COVID-19”, Acta Biomedica 2020; 91(2): 239-241.
4. Sofosbuvir Drug profile, January 2014, http://www.drugbank.ca/drugs/DB08934. Access on 14/06/2022.
5. Centr.al Drugs Standard Control Organization (CDSCO) list of approved drugs 2015. Access on 14/06/2022.
6. Mastanamma SK, Chandini SK, Reehana SK, Saidulu P. Development and validation of stability indicating RP-HPLC method for the simultaneous estimation of Sofosbuvir and Ledipasvir in bulk and their combined dosage form. Futur Journal of Pharmaceutical Sciences. 2018; 4(2):116–23.
7. Mohamed El-Kassem M Hassouna, Maha Mohammed Abdelrahman and Mahmoud Abdel fatah Mohamed, “Assay and Dissolution Methods Development and Validation for Simultaneous Determination of Sofosbuvir and Ledipasvir by RP-HPLC Method in Tablet Dosage Forms”, Journal of Forensic Sciences & Criminal Investigation 2017; 1(3): 001- 010.
8. Jahnavi bandla, S. Ganapaty, “ Development and Validation of a Stability-indicating Method for the Simultaneous Estimation of Sofosbuvir and Ledipasvir by RP-HPLC”, Indian Journal of Pharmaceutical Sciences 2018; 80(6):1170-1176.
9. K. Swathi, P. Venkateswara Rao, N. Srinivasa Rao, “A New Analytical Method for Determination of Ledipasvir and Sofosbuvir in Pharmaceutical Formulations by HPLC Method” International Journal of Research in Pharmaceutical Analysis 2019: 1(3): 59- 67.

10. Bakht Zaman, Faisal Siddique, Waseem Hassan, “RP-HPLC Method for Simultaneous Determination of Sofosbuvir and Ledipasvir in Tablet Dosage Form and Its Application to In Vitro Dissolution Studies”, Chromatographia 2016; 79: 1605- 1613.
11. Naik Desai Prabhat Prabhaker, Kapupara Pankaj. Development and Validation of RP-HPLC Method for the estimation of Sofosbuvir and Ledipasvir in combined dosage form. International Journal of Research in Pharmaceutical Sciences. 2021; 12(1):523–9.
12. Rote AP, Alhat J, Kulkarni AA. Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Ledipasvir and Sofosbuvir in Bulk and Pharmaceutical Dosage Form. International Journal of Pharmaceutical Sciences and Drug Research. 2017 20; 9(06).
13. Sharma S, Goyal S, Chauhan K. A review on analytical method development and validation. Vol. 10, International Journal of Applied Pharmaceutics. Innovare Academics Sciences Pvt. Ltd; 2018. p. 8–15.
14. ICH guidelines Q2 R1 International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Geneva, 2006 www.ich.co.in. Access on 14/06/2022
15. Current Good Manufacturing Practices for finished Pharmaceuticals, 21 CFR, Part 210 and 211, US Food and Drug Administration. Access on 24/06/2022