International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.10 No.7, pp 761-768, 2017

A New RP-HPLC Method for the Simultaneous Assay of SOFOSBUVIR and LEDIPASVIR in Combined Dosage Form

T. Nagaraju[1], S.V.M.Vardhan[2]*, D.Ravi Kumar[3] and D.Ramachandran[4]

[1,3] Department of Chemistry,Krishna University Dr.MRAR Post Graduate Centre,Nuzvid,A.P,India
[2] Department of Biochemistry,Krishna University Dr.MRAR Post Graduate Centre,Nuzvid,A.P,India
[4]Department of Chemistry, Acharya Nagarjuna Univesity,Guntur,A.P,India

Abstract : A new RP-HPLC method was developed for the simultaneous assay of sofosbuvir and ledipasvir in combined dosage form, using Inertsil ODS column (Make: 150 mmx4.6 mm I.D; particle size 5µm and a mobile phase composed of TFA- Buffer(pH -2.0), Acetonitrile and Methanol (30:50:20% v/v/v) at a flow rate of 1.0mL/min. The retention times of sofosbuvir and ledipasvir were found to be 3.205 and 3.774 min, respectively. Linearity was established for sofosbuvir and ledipasvir in the concentration ranges of 40-120ìg/ml and 10-30ìg/ml, respectively. Regression analysis showed a correlation coefficient of greater than 0.999 for sofosbuvir and ledipasvir. The percentage recoveries of sofosbuvir and ledipasvir were found to be in the range of 99.2 to 100.9% and 98.40 to 100.9% respectively. This proposed RP-HPLC method can be successfully employed for simultaneous quantitative analysis of sofosbuvir and ledipasvir in various combined formulations available in the local pharmacies.
Keywords : Sofosbuvir, Ledipasvir and Validation.

Introduction

Sofosbuvir[1,2] Fig.1.(propan-2-yl (2S)-2-{[(S)-{[(3R,4R,5R) -5-(2,4-dioxo-1,2,3,4-tetrahydro pyrimidin-1-yl) -4-fluoro-3-hydroxy-4-methyloxolan-2-yl]methoxy phenoxy) phosphoryl] amino} propanoate) is a prodrug nucleotide analog used as part of combination therapy to treat hepatitis C virus (HCV) infection or to treat co-infection of HIV and HCV. It has a molecular formula of $C_{22}H_{29}FN_3O_9P$ and a molecular weight of 529.45.After metabolism to the active antiviral agent 2'-deoxy-2'-α-fluoro-β-C-methyluridine-5'-triphosphate (also known as GS-461203), the triphosphate serves as a defective substrate for the NS5B protein, an RNA-dependent RNA polymerase required for replication of viral RNA.

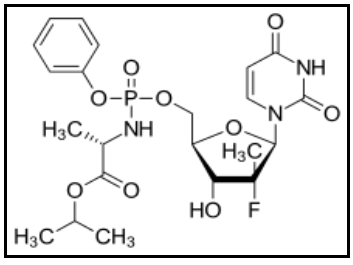


Figure.1.Chemical structure of Sofosbuvir

Ledipasvir[3] Fig.2. ((2S)-1-[(6S)-6-[5-(9,9-difluoro -7-{2-[(1R, 3S, 4S)-2-[(2S)-2-{[hydroxyl (methoxy) methylidene] amino}-3-methyl butanoyl]-2-azabicyclo [2.2.1] heptan-3-yl] -1H-1,3 -benzodiazol-6-yl}-9H-fluoren-2-yl) -1H-imidazol-2-yl]-5-azaspiro[2.4] heptan-5-yl]-2-{[hydroxyl (methoxy) methylidene] amino}-3-methyl butan-1-one) is a Hepatitis C Virus NS5A Inhibitor with potential activity against HCV. In combination with sofosbuvir for treatment in chronic hepatitis C genotype 1 patients. Upon oral administration and after intracellular uptake, ledipasvir binds to and blocks the activity of the NS5A protein. This results in the disruption of the viral RNA replication complex, blockage of HCV RNA production, and inhibition of viral replication. NS5A, a zinc-binding and proline-rich hydrophilic phosphoprotein, plays a crucial role in HCV RNA replication.

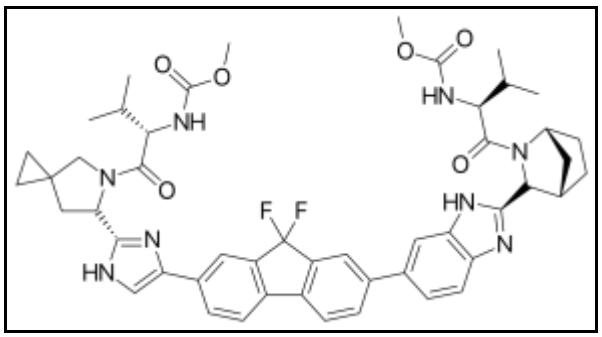


Figure.2.Chemical structure of Ledipasvir

Combination of these two drugs [4-7] (Sofosbuvir 400mg + Ledipasvir 90mg:is available in local pharmacy under the brand name of **MyHep LVIR, LEDIFOS TABLET, HETEROSOFIR PLUS** and **HEPCINAT LP TABLET** etc. The mechanism action involves that sofosbuvir inhibits the RNA polymerase that the Hepatitis C virus uses to replicate its RNA and where as ledipasvir inhibits an important viral phosphoprotein, ns5a, which is involved in viral replication, assembly, and secretion.

According to the best of our knowledge, only three HPLC methods[8-10] have been published, during the preparation of the present work for publishing. The present study aimed to develop a simple, sensitive, short retention time and accurate RP-HPLC method for the simultaneous determination of both sofosbuvir and ledipasvir together in pure and tablet dosage forms with high sensitivity, selectivity that can be used for the routine analysis of production samples.

Materials and methods

Instrumentation

The present assay was carried out on a Waters HPLC system [Model: 2695] equipped with 2487 photodiode array detector, automated sample injector and a column Inertsil ODS(150mmx4.6mm I.D;particle size 5µm) respectively. Electronic Balance [SAB224CL;SCALETEC] and Ultra-Sonicator [SE60US; ENERTECH] were also used in the present assay. The output of signal was monitored and integrated using waters Empower 2 software.

Chemicals and Reagents

Pure standard samples of sofosbuvir and ledipasvir were obtained as gifted samples from Hetero Healthcare Ltd. and its marketed formulations in the brand name of **Heterosofir Plus** [Label claim containing sofosbuvir 400mg and ledipasvir 90 mg] were procured from local pharmacy. Trifluoroacetic acid (HPLC-Grade;Qualigens), Water(HPLC-Grade), Acetonitrile(HPLC-Grade;Qualigens) and Methanol (HPLC-Grade Rankem). All dilutions were performed in standard class-A, volumetric glassware.

Buffer preparation

Dissolve 1.0g of Trifluoroacetic acid (HPLC-Grade;Qualigens),in 1000mL of Water, and filter the solution through 0.45µm membrane filter.

Mobile phase preparation:

Prepare a filtered and degassed mixture of Buffer(pH -2.0), Acetonitrile and Methanol (30:50:20% v/v/v) respectively.

Diluent preparation

Mobile phase is used as diluent.

Standard preparation:

About 100mg of sofosbuvir and ledipasvir were accurately weighed and taken separately in 100ml volumetric flasks separately and dissolved in the mobile phase. Solutions were sonicated for 5mins. The volume was adjusted to the mark with diluent to obtain stock solution of concentration 1.0mg/ml of sofosbuvir and ledipasvir separately. Calibration standards were prepared using the stock solutions [40-120µg/ml of sofosbuvir and 10- 30µg/ml of sofosbuvir].

Sample preparation

Ten tablets of **Heterosofir Plus** [Label claim containing sofosbuvir 400mg and ledipasvir 90 mg] were weighed and finely powdered in a pestle and mortar. Tablets powder equivalent to 100mg of sofosbuvir and ledipasvir was transferred to 100ml volumetric flask and dissolved in about 50ml of mobile phase. The solutions were sonicated for 15min., diluted to the mark with mobile phase and then filtered through 0.45µm membrane filters (Millipore, USA). Aliquots of the sample solution were transferred to 50 ml volumetric flasks and diluted with diluent to obtain concentration of 40-120µg/ml of sofosbuvir and 10- 30µg/ml of ledipasvir respectively.

Results and Discussion

Method development

Initial trials were carried by the author in developing the proposed RP-HPLC method. The mobile phase was chosen after several trials with methanol, acetonitrile, water and buffer solutions in various proportions and at different pH values. A mobile phase consisting of buffer (pH -2.0), acetonitrile and methanol (30:50:20% v/v/v) was selected to achieve maximum separation and sensitivity. Flow rates between 0.5 and 1.5/min were studied. A flow rate of 1.0 ml/min at ambient temperature gave an optimal signal to noise ratio with a reasonable separation time. Using a C18 column, the retention times for sofosbuvir and ledipasvir were observed to be 3.205 and 3.774 min, respectively. Total time of analysis was less than 5 min. Detection wavelength of 267nm was chosen for the analysis (Figure 2).A typical chromatogram for simultaneous estimation of sofosbuvir and ledipasvir obtained by using the aforementioned mobile phase from 10µL injection volume of the assay preparation is illustrated in Fig.3**.**

Chromatographic conditions

The isocratic mobile phase consisted of buffer (pH -2.0), acetonitrile and methanol (30:50:20% v/v/v), flowing through the Inertsil ODS C_{18} column (make: 150 mmx4.6 mm i.d; particle size 5µm) at a constant flow rate of 1.0 ml/min at ambient column temperature. The mobile phase was pumped through the column at a flow rate of 1.0ml/min with a sample injection volume of 10µl. Detection of the analytes (sofosbuvir and ledipasvir) were carried out at a wavelength of 267 nm.

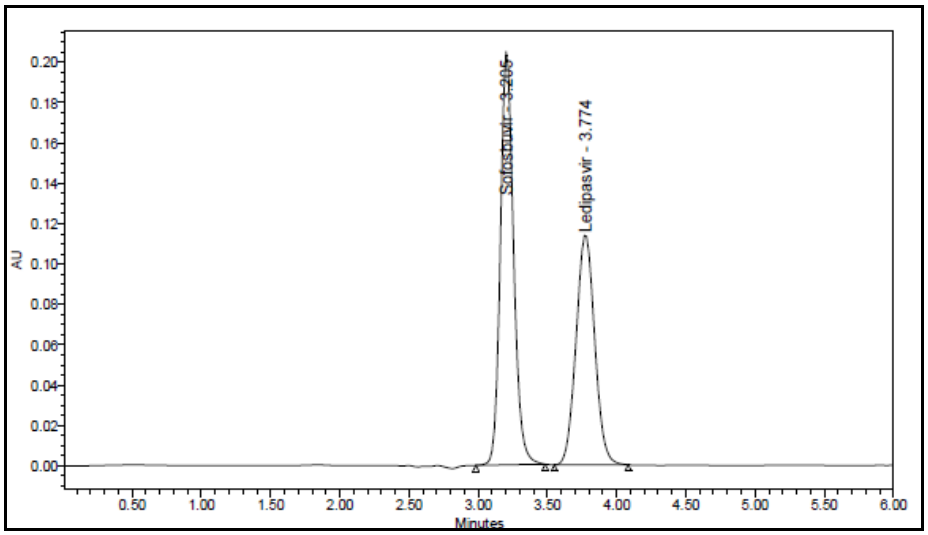


Figure-3Typical Chromatogram of Standard solution(sofosbuvir and ledipasvir)

Method validation

The proposed RP-HPLC method was validated, in accordance with USP guidelines for system suitability, linearity, LOD & LOQ, precision, accuracy, specificity, ruggedness and, robustness, [26**]** spectively.

System suitability

System performance parameters of the proposed RP-HPLC method were determined by analyzing standard working solutions of sofosbuvir and ledipasvir. The chromatographic parameters, such as number of theoretical plates (n), resolution (rs), USP plate count and USP tailing were determined. The results are shown in Table 1, indicating the good performance of the system.

Table 1: System Suitability Parameters of Sofosbuvir and Ledipasvir

System suitability data of Sofosbuvir and Ledipasvir					
S.no	**Sample name**	**RT**	**Area**	**USP plate count**	**USP tailing**
1.	Injection1	3.205	1356838	5243	1.15
System suitability data of Ledipasvir					
S.no	**Sample name**	**RT**	**Area**	**USP plate count**	**USP tailing**
1.	Injection 1	3.774	1068182	3630	1.05

Specificity

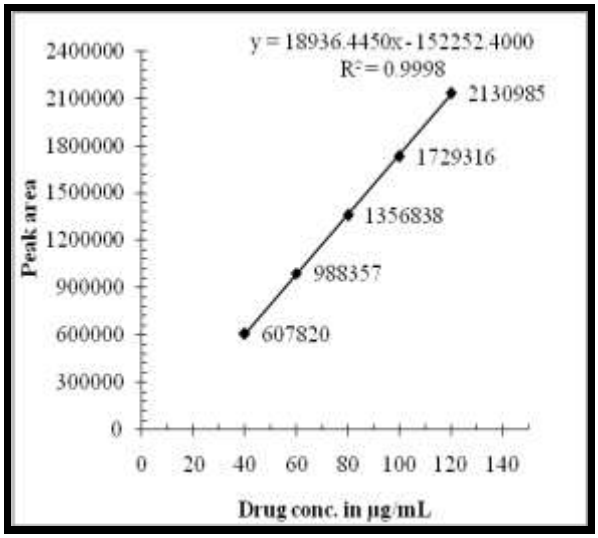
Blank and placebo interference:

The interference of blank and placebo with the elution of the present cited drugs solutions of diluent and placebo were injected into the chromatographic system with the mentioned chromatographic conditions and their respective chromatograms were recorded. From the reported chromatograms it was observed that the placebo and blank showed no peaks at the retention time of sofosbuvir and ledipasvir peak indicating that the diluent and placebo solutions used in standard and sample preparations did not interfered in assay of sofosbuvir and ledipasvir respectively.

Linearity & Detector response

The linearity of the proposed method was accessed by calculating slope, intercept and correlation coefficient [r2] of standard curve. Sofosbuvir and ledipasvir showed a linearity of response between 40-120 and 10-30µg/ml, respectively and the slope and intercept of the calibration plot of sofosbuvir and ledipasvir were **18936.4x-152252.4** and **59353.4x - 109982** with correlation coefficients obtained was greater than 0.999 respectively.
The linearity curves of sofosbuvir and ledipasvir were depicted in Figures.4 & 5 and the linearity results of both the drugs were given in Table-2.

The limit of detection (LOD) and limit of quantification (LOQ) were established at signal-to noise ratio of 3:1 and 10:1 respectively. The LOD of sofosbuvir and ledipasvir was found to be 0.015µg /ml & 0.012µg /ml respectively. The LOQ of sofosbuvir and ledipasvir was found to be 0.05 µg /ml & 0.042 µg / ml respectively (Table 2).



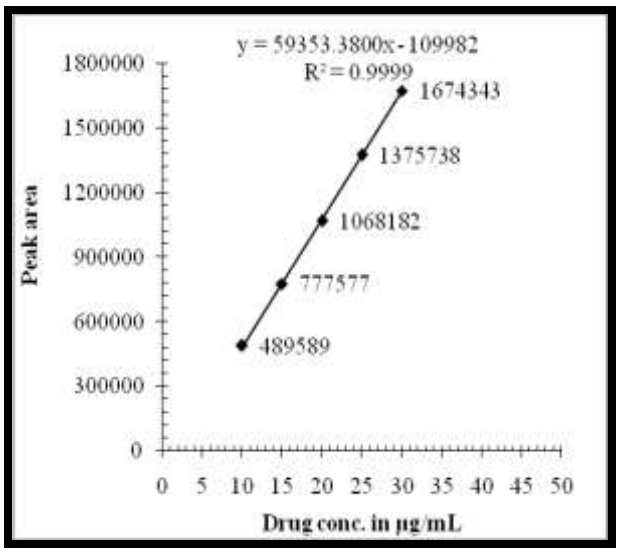


Figure.4.Linear calibration plot of sofosbuvir **Figure.5.Linear calibration plot of ledipasvir**

Table 2: Results of Linearity Studies of Sofosbuvir and Ledipasvir

Sofosbuvir			**Ledipasvir**		
% Level (Approx.)	**Conc. µg/mL**	**Area**	**% Level (Approx.)**	**Conc. µg/mL**	**Area**
50	40	607820	50	10	489589
75	60	988357	75	15	777577
100	80	1356838	100	20	1068182
125	100	1729316	125	25	1375738
150	120	2130985	150	30	1674343
Slope		**18936.4**	**Slope**		**59353.38**
RSQ(r2)		**0.9998**	**RSQ(r2)**		**0.9999**
LOD (µg/mL)		**0.015**	**LOD (µg/mL)**		**0.012**
LOQ (µg/mL)		**0.050**	**LOQ (µg/mL)**		**0.042**

Precision

The precision of sofosbuvir and ledipasvir by proposed RP-HPLC method was ascertained by replicate analysis of homogeneous samples of capsule powder. Intermediate precision of the present RP-HPLC method was studied by intra-day variation of the method was carried out. The results were given in Table 3 and the low % RSD values of within a day for sofosbuvir and ledipasvir revealed that the proposed method is highly precise.

Table 3: Results of Method Precision (Intraday) Studies for Sofosbuvir and Ledipasvir

Sofosbuvir				Ledipasvir		
S.no	**RT**	**Area**	**%Assay**	**RT**	**Area**	**%Assay**
Injection1	3.215	1359925	100.9	3.789	1071854	98.4
Injection2	3.219	1368686	100.3	3.781	1076741	100.3
Injection3	3.236	1362850	99.8	3.799	1077984	100.6
Injection4	3.220	1360509	100.9	3.782	1074216	100.2
Injection5	3.225	1397712	99.0	3.786	1104120	99.2
Injection6	3.224	1371128	100.7	3.787	1080627	100.1
Mean*			**100.3**	**Mean***		**99.8**
Std. Dev.*			**0.77**	**Std. Dev.***		**0.84**
% RSD*			**0.77**	**% RSD***		**0.84**

*Average of six determinations

Accuracy

The accuracy of the proposed method for sofosbuvir and ledipasvir was assessed by recovery studies at three different levels i.e. 50%, 100%, 150%. The recovery studies were carried out in triplicate by adding Known amount of standard solution of sofosbuvir and ledipasvir to preanalysed tablet solutions. The resulting solutions were then reanalysed by proposed method and the results are represented in Table 4. The percentage recoveries were found in the range of 99.2 to 100.9% for sofosbuvir and 98.40 to 100.9% for ledipasvir respectively revealing that the developed RP-HPLC method was found to be accurate.

Table 4: Results of Accuracy Studies for Sofosbuvir and Ledipasvir

S.NO	Accuracy level	Injection	Sofosbuvir	Ledipasvir
			Sample area	**Sample area**
1	**50%**	**1**	**99.2**	**99.9**
		2	**99.6**	**99.7**
		3	**99.6**	**99.5**
2	**100%**	**1**	**100.9**	**98.4**
		2	**100.3**	**100.3**
		3	**99.8**	**100.6**
3	**150%**	**1**	**99.9**	**100.4**
		2	**99.7**	**99.6**
		3	**99.8**	**100.9**

*Average of three determinations

Robustness

The robustness of the developed method was evaluated by altering few experimental conditions and evaluating the resolution between two adjacent peaks of sofosbuvir and ledipasvir. The altered experimental conditions carried out in this study are given below.

Change in flow rate:

The flow rate of the mobile phase was 1.0 ml/min. to study the effect of the flow rate on the resolution, the flow rate was changed by 0.2 units (0.8 and 1.2 ml/min).

Change in column temperature:

The effect of the column temperature on the resolution of sofosbuvir and ledipasvir was studied at 20 ˚C and 30 ˚C instead of 25 ˚C.

in all the above said varied chromatographic conditions (Flow rate and Column temperature) insignificant differences in peak areas and in retention time were observed for sofosbuvir and ledipasvir illustrating the robustness of the developed method (Table 5).

Table 5: Robustness Studies of Sofosbuvir and Ledipasvir

Sofosbuvir			**Ledipasvir**		
Parameter	**RT**	**Area**	**Parameter**	**RT**	**Area**
Decreased flow rate(0.8ml/min)	4.006	1689378	**Decreased flow rate (0.8ml/min)**	4.716	1334441
Increased flow rate(1.2ml/min)	2.685	1125531	**Increased flow rate (1.2ml/min)**	3.159	886509
Change in column temperature at 20 ˚C	4.006	1689378	**Change in column temperature at 20 ˚C**	4.716	1334441
Change in column temperature at 30 ˚C)	2.685	1125531	**Change in column temperature at 30 ˚C**	3.159	886509

Intermediate precision (Ruggedness)

Intermediate precision (Ruggedness) expresses within-laboratories variations: different days, different analysts, different equipments, etc. Good results were obtained and presented in Table 6.

Table 6 : Results of Ruggedness Studies of Sofosbuvir and Ledipasvir

Sofosbuvir			**Ledipasvir**	
S.No.	**Rt**	**Area**	**Rt**	**Area**
1	3.217	1359875	3.787	1071804
2	3.216	1358578	3.779	1076790
3	3.236	1365670	3.780	1077576
4	3.220	1360509	3.784	1076716
5	3.225	1373423	3.785	1089127
6	3.224	1361543	3.789	1080767
***Average**	**3.223**	**1363266**	**3.784**	**1078797**
%RSD*	**0.227**	**0.405**	**0.103**	**0.535**

*Average of five determinations

Formulation assay

The validated method was applied on commercially available Heterosofir Plus. The results of the assay undertaken yielded 99.99% and 99.98% of the label claim for sofosbuvir and ledipasvir. Results of the assay indicated that the method is quite selective for the analysis of Heterosofir Plus ® FCT without interference from the excipients used to formulate and produce these tablets. The results were displayed in Table 7 respectively.

Table 7: Assay of Sofosbuvir And Ledipasvir In Formulations

Drug name [Heterosofir Plus ® FCT]	**Quantity label claim(mg)**	***Quantity found ± SD**	**% Assay ± SD**
Sofosbuvir	4000mg	**399.97 ± 0.87**	**99.99± 0.79**
Ledipasvir	90mg	**89.99 ± 0.69**	**99.98± 0.78**

*Average of three determinations

Conclusions

The proposed reverse phase HPLC method of was validated and applied for the determination of sofosbuvir and ledipasvir in drug forms. The developed RP-HPLC method offered several advantages in terms of economical, simplicity in mobile phase and sample preparation steps. The short run time made the proposed RP-HPLC method more specific, repeatable and reliable for its intended use in assaying of sofosbuvir and ledipasvir in combined dosage forms. From the validation results it is concluded that this proposed method can be used for regular routine analysis and, stability studies.

Acknowledgments

The authors thank Hetero Health Care Pvt Ltd, India, for providing gift sample of standards sofosbuvir and ledipasvir and Dept. of Chemistry, Acharya Nagarjuna University,Guntur for providing facilities for carrying out this study.

References:

1. Keating G.M.,2014. Sofosbuvir: A review of its use in patients with chronic hepatitis C. Drugs. 74(10), pp.1127-1146.
2. Kirby B.J., Symonds W.T., Kearney B.P., Mathias A.A., 2015. Pharmacokinetic Pharmacodynamic and Drug-Interaction Profile of the Hepatitis C Virus NS5B Polymerase Inhibitor Sofosbuvir. Clinical Pharmacokinetics.54 (7),pp.677-690.
3. Link J.O., Taylor J.G., Xu L et al, 2014. Discovery of ledipasvir (GS-5885): A potent, once-daily oral NS5A inhibitor for the treatment of hepatitis C virus infection. Journal of Medicinal Chemistry. 57, pp.2033-2046.
4. Kumari R., Nguyen M.H., 2015. Fixed-dose combination of sofosbuvir and ledipasvir for the treatment of chronic hepatitis C genotype 1. Expert Opinion on Pharmacotherapy, 6(5), pp.739-748.
5. Younossi Z.M., Stepanova M., Marcellin P., Afdhal N., Kowdley K.V., Zeuzem S., Hunt S.L., 2015.Treatment with ledipasvir and sofosbuvir improves patient-reported outcomes: Results from the ION-1, -2, and -3 clinical trials. Hepatology. ,61(6),pp.1798-808.
6. Waheed Y., 2015. Ledipasvir and sofosbuvir: Interferon free therapy for hepatitis C virus genotype 1 infection. World Journal of Virology. 2015, 12;4(1),pp.33-5.
7. Osinusi A., Townsend K.., Kohli A., Nelson A., Seamon C., Meissner E.G., Bon D., Silk R., Gross C., Price A., Sajadi M., Sidharthan S., Sims Z., Herrmann E., Hogan J., Teferi G., Talwani R., Proschan M., Jenkins V., Kleiner D.E., Wood B.J., Subramanian G.M., Pang PS, Mc Hutchison J.G., Polis M.A., Fauci A.S., Masur H, Kottilil S., 2015. Virologic response following combined ledipasvir and sofosbuvir administration in patients with HCV genotype 1 and HIV co-infection. Journal of the American Medical Association. 313(12),pp.1232-1239.
8. Bakht Zaman,Faisal Siddique.,Waseem Hassan..,2016.RP-HPLC method for simultaneous determination of sofosbuvir and ledipasvir in tablet dosage form and its application to in vitro dissolution studies.Chromatographia.79(23),pp.1605–1613.
9. Rezk M.R., Bendas E.R, Basalious E.B., Karim I.A.,2016.Quantification of sofosbuvir and ledipasvir in human plasma by UPLC-MS/MS method: Application to fasting and fed bioequivalence studies. J Chromatogr B Analyt Technol Biomedical Life Sciences. 1028,pp.63-70
10. Hassouna M. E. M., Abdelrahman M. M., Mohamed M. A., 2017. 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 Science & Criminal Investigations. 1(3),pp.555-562.
11. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH harmonised tripartite guideline: validation of analytical procedures: text and methodology Q2(R1). ICH. 2005; 1-13.

*****