# Development and validation for the simultaneous estimation of Sofosbuvir and Ledipasvir by UV spectrophotometer method in bulk and tablet dosage forms

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## ABSTRACT

**Objective:** In the present work, A Simple, rapid, sensitive, precise and reproducible specific UV spectrophotometric method for the determination of Sofosbuvir (SOFO) and Ledipasvir (LEDI) in bulk drug and pharmaceutical dosage form were developed and validated**. Methods:** A simple double beam UV spectrophotometric method has been developed and validated with different parameters such as linearity, precision, repeatability, limit of detection (LOD), Limit of Quantification (LOQ), accuracy as per ICH guidelines. **Results:** UV-visible Spectrophotometric method, measurement of absorption at maximum wavelength in 10 ml methanol and volume make with water solvent system as reference Sofosbuvir and Ledipasvir were found to be at 250 nm and 333 nm respectively. The drug obeyed the Beer's law and showed good correlation. Beer's law was obeyed in concentration range 8-40 µg/ml for Sofosbuvir and 2-10µg/ml for Ledipasvir respectively with correlation coefficient was 0.998. The LOD and LOQ of Sofosbuvir was found to be 0.047 µg/ml and 0.142 µg/ml, Ledipasvir were found to be 1.02 µg/ml and 3.11 µg/ml, respectively. Percentage assay of SOFO and LEDI in tablets. **Conclusion:** The proposed method is simple, precise, accurate and reproducible can be used for routine analysis of Sofosbuvir and Ledipasvir in bulk and tablet dosage form.

**Keywords:** Sofosbuvir, Ledipasvir, Method development, Validation, UV spectrophotometric, Dosage forms.

## INTRODUCTION

Globally, 130-150 millions of people have chronic hepatitis C infection. A significant number of those who are chronically infected will develop liver cirrhosis or liver cancer. Gilead Sciences overcome most common related liver diseases by its Great invention (Harvoni). Harvoni (90 mg ledipasvir/400 mg sofosbuvir) approved by United States FDA. (Harvoni, 2016; Gilead Files, 2014) [1-2]. Chronic hepatitis C virus (HCV) infection is one of the most common etiologies of liver-related mortality throughout the world. Among the six HCV genotypes, genotype 1 was significantly more aggressive Among the

available treatments for HCV genotype 1, the combination therapy of ledipasvir/sofosbuvir provides several advantages compared to other regimens, including use of a single-pill regimen, possibility to shorten the duration of treatment to 8 weeks, efficacy in patients exposed to protease inhibitors, safety in decompensated cirrhosis, and potential to avoid ribavirin [3-5]. Sofosbuvir and ledipasvir are inhobits HCV NS5B and HCV NS5A polymerase respectively. A fixed-dose combination of sofosbuvir–ledipasvir was approved in 2014 for treatment of patients chronically infected with genotype 1HCV[6].

Sofosbuvir (SOF); is chemically known as (S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl) methoxy)-(phenoxy) phosphorylamino) propanoate . It has a molecular formula of $C_{22}H_{29}FN_3O_9P$ and a molecular weight of 529.45 **(Figure 1).**

Sofosbuvir is a white to off-white powder with a solubility of ≥ 2 mg/mL across the pH range of 2-7.7 at 37°C. The partition coefficient (log P) for Sofosbuvir is 1.62 and the pKa is 9.3 [7]. Sofosbuvir is a pangenotypic inhibitor of the HCV NS5B RNA-dependent RNA polymerase, which is essential for viral replication [8]. Sofosbuvir is a nucleotide prodrug that undergoes intracellular activation to form GS-461203 (active triphosphate, not detected in plasma), and ultimately the inactive, renally eliminated metabolite GS-331007 [9]. The pharmacologically active uridine analog triphosphate (GS-461203) can be incorporated by HCV NS5B and acts as a chain terminator. In a biochemical assay, GS-461203 inhibits the polymerase activity of the recombinant NS5B from HCV genotype 1b, 2a, 3a and 4a with an IC50 value ranging from 0.7 to 2.6 µM. GS-461203 is neither an inhibitor of human DNA and RNA polymerases nor an inhibitor of mitochondrial RNA polymerase [10]. Sofosbuvir/Ledipasvir is a fixed-dose combination (FDC) tablet containing Sofosbuvir (a previously approved NS5B polymerase inhibitor) and ledipasvir, a new NS5A-inhibitor [7].

Ledipasvir (LDV); is chemically known as Methyl [(2S)-1-{(6S)-6-[5-(9,9-difluoro-7- {2-[(1R,3S,4S)-2-{(2S)-2-[(methoxycarbonyl)amino]-3-methylbutanoyl}-2-azabicyclo[2.2.1] hept-3-yl]-1H-benzimidazol-6-yl}-9H-fluoren-2-yl)-1H-imidazol-2-yl]-5-azaspiro[2.4] hept-5-yl}-3-methyl-1-oxobutan-2-yl] carbamate. It has a molecular formula of $C_{49}H_{54}F_2N_8O_6$ and a molecular weight of 889.00 **(Figure 2).** Ledipasvir is a white to tinted (off-white, tan, yellow, orange, or pink), slightly hygroscopic crystalline solid. Ledipasvir is practically insoluble (<0.1 mg/mL) across the pH range of 3.0-7.5 and is slightly soluble below pH 2.3 (1.1 mg/mL). The partition coefficient (log P) for ledipasvir is 3.8 and the pKa1 is 4.0 and pKa2 is 5.0 [7]. Ledipasvir is an HCV inhibitor targeting the HCV NS5A protein, which is essential for both RNA replication and the assembly of HCV virions. Biochemical confirmation of NS5A inhibition of ledipasvir is not currently possible as NS5A has no enzymatic function. In vitro resistance selection and cross-resistance studies indicate ledipasvir targets NS5A as its mode of action [10]. The combination of these two drugs is not official in any pharmacopoeia [11-12]. Very recently, a limited number of methods have been developed for the individual and simultaneous determination of both drugs. The degradation products of SOFO under several stress

conditions have been determined by HPLC [13-14]. SOF's disposition was characterized into various in vivo cell types [15].

Sofosbuvir in human plasma was determined by UPLC-MS/MS method [16]. Quantification of Sofosbuvir and its metabolite, GS-331007, in human plasma has been determined by UPLC-ESI-MS/MS method [17]. Simultaneous quantification of ribavirin, sofosbuvir and its metabolite in rat plasma by UPLC-MS/MS has been reported [18]. SOFO in pure form [19], in bulk and tablet dosage form was determined by RP-HPLC [20]. Finally, sofosbuvir (SOF) was used as an internal standard (IS) in an UPLC-MS/MS method for the determination of daclatasvir (DAC) in human plasma [21]. While for LDV, only two methods have been published for its individual determination in bulk drug form by simple UV spectrophotometry [22] and by RP-HPLC [23]. Both sofosbuvir and ledipasvir in human plasma were determined by UPLC-MS/MS method [24] and besides some antiviral agents [25]. Ledipasvir, sofosbuvir and its metabolite in rat plasma were also, determined by UPLC-MS/MS [26].

According to the best of our knowledge, only two HPLC methods [22] 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 UV spectrophotometry 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.

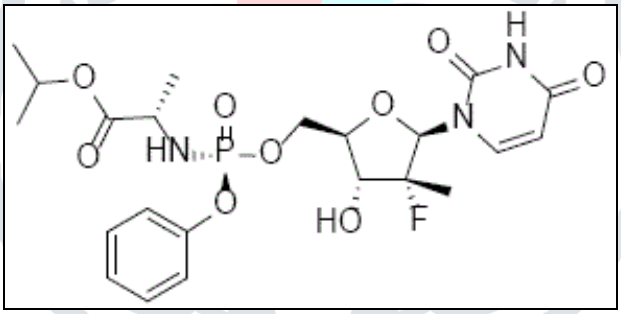


**Fig. 1: Structure of Sofosbuvir**

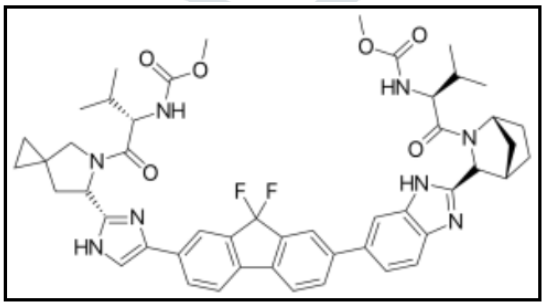


**Fig. 2: Structure of Ledipasvir**

## MATERIALS AND METHODS

### Instrument

A Shimadzu UV/Visible double beam spectrophotometer (Model 1700) with 1cm matched quartz cells was used in present study for multi component analysis.

**Materials and Reagents**

Sofosbuvir and Ledipasvir were obtained as gift samples from R.S.I.T.C Jalgaon. O-phosphoric acid were procred from Avantor Performance material India Ltd. Thane, Maharashtra and Methanol were HPLC grade procured from Merck specialities Pvt. Ltd. Shiv Sager Estate 'A' Worli, Mumbai. A combination of Sofosbuvir (400 mg) and Ledipasvir (90 mg) in tablet formulation was procured from Hetero drugs Ltd. Mumbai (Ledifos brand).

**Preparation of standard stock solution:**

**Sofosbuvir standard stock solution : (Stock I )**

An accurately weighed quantity, 40 mg of SOFO was dissolved in Methanol in a 10 ml volumetric flask and volume made up to 10.0 ml to produce a solution of 4000 µg/ml **(Fig. 3).**

**Ledipasvir standard stock solution: (Stock II)**

An accurately weighed quantity, 10 mg of LEDI was dissolved in Methanol in 10 ml volumetric flask and volume made up to 10.0 ml to produce a solution of 1000 µg/ml **(Fig. 4)**.

**Preparation of Stock Standard Combination Solution : ( Stock III) [SOFO + LEDI]**

Accurately weight and transfer 40mg Sofosbuvir and Ledipasvir10mg working standard into 10 ml volumetric flask as about diluent Methanol completely and make volume up to the mark with the same solvent to get 1000µg/ml standard (stock solution) and 15 min sonicate to dissolve it and remove the unwanted gas further an aliquots portion of Sofosbuvir and Ledipasvir stock solution in ratio of 80:20were mixed in volumetric flask in 10 ml and volume was adjusted up to mark with mobile phase from the resulting solution 0.1ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with Acetonitrile :Water (0.1% OPA with TEA), prepared in (8ml Acetonitrile: 2ml Water (0.1% OPA) solvent. Result as shown **(Fig. 5).**

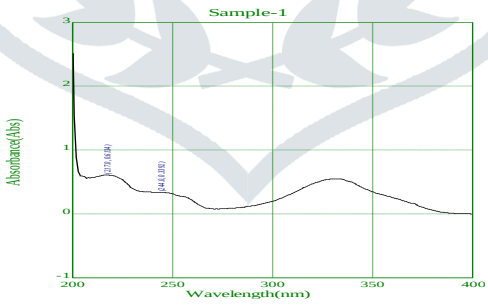


**Fig. 3: UV Spectrum of Sofosbuvir**

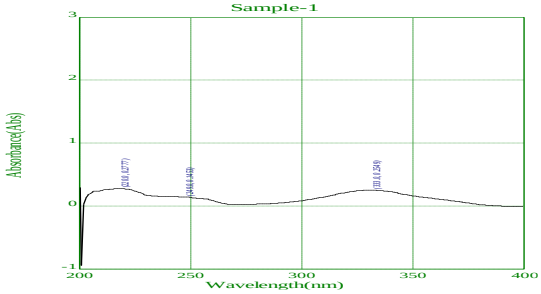


**Fig. 4: UV Spectrum of Ledipasvir**

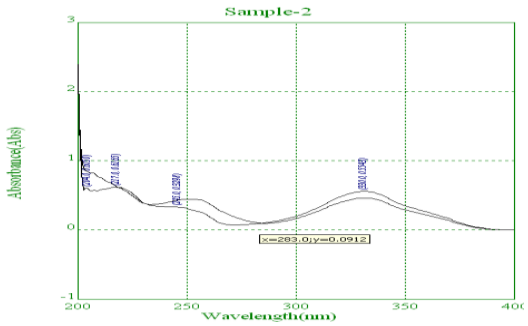


**Fig. 5: Iso-absorptive point of Sofosbuvir and Ledipasvir**

**Assay preparation for marketed formulation**

For analysis of the tablet dosage form, weigh 20 Sofosbuvir and Ledipasvir combination tablets and calculated the average weight, accurately weigh and transfer the sample equivalent to 12.2 mg Sofosbuvir and Ledipasvir into 10 ml volumetric flask. Add about 10ml ACN of diluent and sonicate to dissolve it completely and make volume up to the mark with diluent. Mix well and filter through 0.45 µm nylon membrane filter. Then volume was made up to the mark with Acetonitrile + 0.1% OPA water with TEA(80 + 20% v/v). The simple chromatogram of test Sofosbuvir and Ledipasvir shown in **(Fig. 6).** The amounts of Sofosbuvir and Ledipasvir per tablet were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated five times with tablet formulation. Tablet Assay for % Label claim for % RSD Calculated, Result was shown in **(Table 1).**

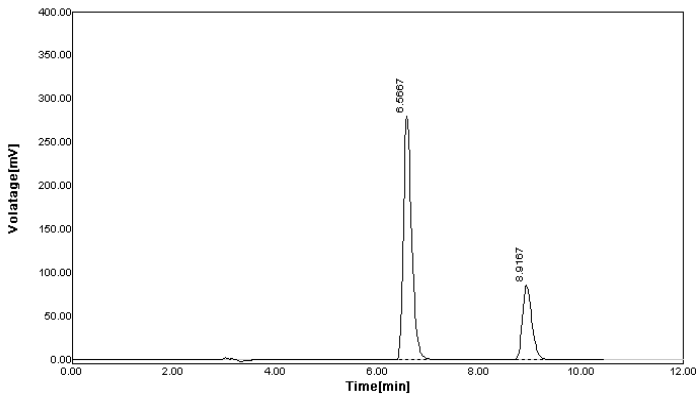


**Fig. 6: Chromatogram for Marketed Formulation**

**Table 1: Analysis of marketed formulation**

| Assay | Drug | Label claimed | Amt. Found | % Label claim | S.D. | %RSD |
|---|---|---|---|---|---|---|
| UV Method | SOFO | 40 | 39.78 | 99.45 | 0.64 | 0.18 |
| | LEDI | 10 | 9.92 | 99.20 | 0.35 | 0.37 |
| | SOFO | 40 | 39.52 | 98.80 | 0.61 | 0.46 |
| | LEDI | 10 | 9.98 | 98.80 | 0.03 | 0.29 |

## METHOD VALIDATION [27-31]

The proposed methods were validated accordance to ICHQ2 (R1) guidelines for linearity, precision, accuracy, limit of detection, limit of quantification.

## RESULTS

### Linearity and range

The mobile phase was allowed to equilibrate with stationary phase until OPA by baseline was obtained. The data obtained in the calibration experiments when subjected to linear regression analysis showed a linear relationship between peak areas and concentrations in the range 8-40µg/mL for Sofosbuvir and 2-10µg/mL for Ledipasvir (**Table 2 and 3)** depict the calibration data of Sofosbuvir and Ledipasvir. The respective linear equation for Sofosbuvir was y = 0.019x+0.104 and Ledipasvir equation y = 0.098x+0.006 where x is the concentration and y is area of peak. The correlation coefficient of Sofosbuvir 0.998 and Ledipasvir was 0.997. The calibration curve of Sofosbuvir and Ledipasvir is depicted in (**Fig. 7 and 8).**

**Table 2: Linearity data for Sofosbuvir**

| Method | Conc. µg/ml | Peak area (µV.sec) | | Average peak area (µV.sec) | S.D. of Peak Area | % RSD of Peak Area |
|---|---|---|---|---|---|---|
| | | 1 | 2 | | | |
| UV Method | 8 | 0.2569 | 0.2511 | 0.25 | 0.004 | 1.61 |
| | 16 | 0.425 | 0.4257 | 0.43 | 0.0005 | 0.12 |
| | 24 | 0.5512 | 0.5499 | 0.55 | 0.001 | 0.17 |
| | 32 | 0.7268 | 0.719 | 0.72 | 0.01 | 1.76 |
| | 40 | 0.8612 | 0.8543 | 0.86 | 0.002 | 0.21 |
| | **Equation** | | y= 0.019x+0.104 | | | |
| | **$R^2$** | | 0.998 | | | |

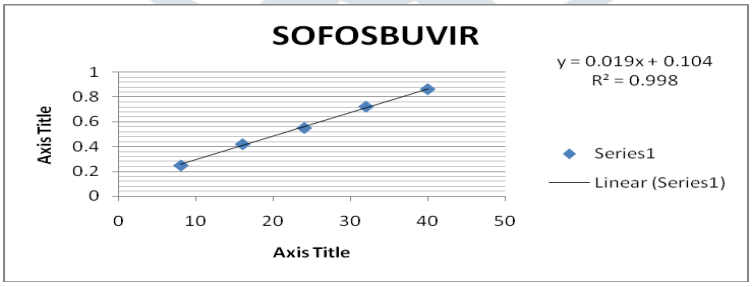


**Fig. 7: Calibration curve of Sofosbuvirfor (UV method)**

**Table 3: Linearity data for Ledipasvir**

| Method | Conc. µg/ml | Peak area(µV.sec) | | Average peak area (µV.sec) | S.D. of Peak Area | % RSD of Peak Area |
|---|---|---|---|---|---|---|
| | | 1 | 2 | | | |
| UV Method | 2 | 0.1938 | 0.1944 | 0.19 | 0.00 | 0.22 |
| | 4 | 0.4035 | 0.4026 | 0.40 | 0.00 | 0.16 |
| | 6 | 0.6259 | 0.6265 | 0.63 | 0.00 | 0.07 |
| | 7 | 0.7801 | 0.79 | 0.79 | 0.01 | 0.89 |
| | 10 | 0.98 | 0.9801 | 0.98 | 0.01 | 0.06 |
| | Equation | | y = 0.098x+0.006 | | | |
| | $R^2$ | | 0.997 | | | |

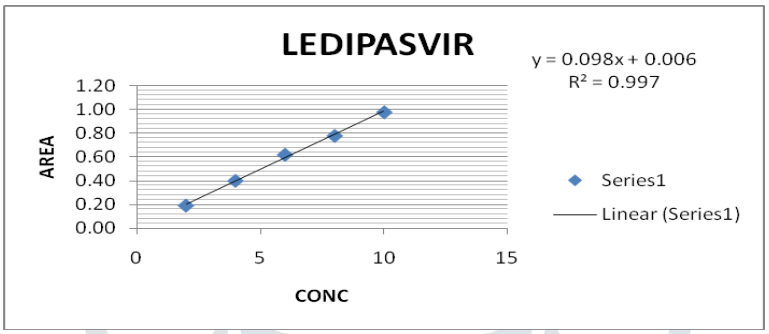


**Fig. 8: Calibration curve of Ledipasvir for (UV method)**

**Accuracy**

Recovery studies were performed to validate the accuracy of developed method. To pre analyzed tablet solution, a definite concentration of standard drug (80%, 100% and 120%) was added and then its recovery was analyzed **Table 4**. Accuracy of UV spectroscopic method were ascertained by recovery studies performed at different levels of concentrations (80%, 100% and 120%). The % recovery was found to be within 98-101%. Statistical validation of recovery studies shown in **Table 5.**

**Table 4: Result of Recovery data for Sofosbuvir and Ledipasvir**

| Drug | Level (%) | Amt. taken (µg/ml) | Amt. Added (µg/ml) | Absorbance Mean* ± S.D. | Amt. recovered Mean*± S.D. | %Recovery Mean*± S.D. |
|---|---|---|---|---|---|---|
| SOFO | 80% | 8 | 6.4 | 0.376± 0.04 | 6.33± 0.04 | 99.09 ±0.56 |
| | 100% | 8 | 8 | 0.4072± 0.06 | 7.96± 0.06 | 99.47±0.83 |
| | 120% | 8 | 9.4 | 0.4395± 0.05 | 9.61± 0.01 | 100.636±0.50 |
| LEDI | 80% | 2 | 1.6 | 0.361± 0.01 | 2.02± 0.01 | 101.17± 0.65 |
| | 100% | 2 | 2 | 0.4038 ± 0.02 | 2.06 ±0.02 | 102.00±1.94 |
| | 120% | 2 | 2.4 | 0.433 ±0.04 | 1.96± 0.04 | 97.84±1.90 |

***mean of each 3 reading for UV method**

**Table 5: Statistical validation of recovery studies Sofosbuvir and Ledipasvir**

| Level of Recovery (%) | Drug | Mean % Recovery | S. D.* | % RSD |
|---|---|---|---|---|
| 80% | **SOFO** | 99.09 | 0.58 | 0.59 |
| | **LEDI** | 101.17 | 0.65 | 0.64 |
| 100% | **SOFO** | 99.47 | 0.83 | 0.84 |
| | **LEDI** | 102.45 | 1.94 | 1.90 |
| 120% | **SOFO** | 100.63 | 0.50 | 0.50 |
| | **LEDI** | 97.84 | 1.90 | 1.94 |

***Denotes average of three determinations for UV method**

**System suitability parameters**

Repeatability studies on UV method for Sofosbuvir and Ledipasvir was found to be, the % RSD was less than 2%, which shows high percentage amount found in between 98% to 102% indicates the analytical method that concluded **Table 6**.

**Table 6: Repeatability studies on UV method for Sofosbuvir and Ledipasvir**

| Method | Conc. of SOFO and LEDI (mg/ml) | Peak area | Amount found (mg) | % Amount found |
|---|---|---|---|---|
| **UV Method for SOFO** | 16 | 0.4012 | 11.84 | 98.66 |
| | 16 | 0.4036 | 11.98 | 98.22 |
| | 16 | 0.4089 | 12.19 | 101.63 |
| | 16 | 0.4025 | 11.94 | 99.50 |
| | 16 | 0.4011 | 11.88 | 99.03 |
| | | **Mean** | **11.97** | **99.41** |
| | | **SD** | **0.14** | **1.33** |
| | | **%RSD** | **1.14** | **1.34** |
| **UV Method for LEDI** | 4 | 0.411 | 4.13 | 102.94 |
| | 4 | 0.4097 | 4.11 | 101.25 |
| | 4 | 0.4082 | 4.10 | 102.60 |
| | 4 | 0.4026 | 4.04 | 101.17 |
| | 4 | 0.4011 | 4.03 | 100.79 |
| | | **Mean** | **4.08** | **101.75** |
| | | **SD** | **0.04** | **0.94** |
| | | **%RSD** | **1.09** | **0.95** |

**Precision**

Precision was studied to find out intra and inter-day variations in the test method of SOFO and LEDI. Intra-day precision was determined by analyzing three concentration in three replicate measurements of within linearity range of drugs on three different times in the same day. Inter-day precision was

conducted during routine operation of the system over a period of 3 consecutive days. Intraday and Inter day Precision studies on UV method for SOFO and LEDI which shows the high precision % amount in between 98% to 100% indicates to analytical method that concluded **Table 7**.

**Table 7: Result of Intra day and Inter day Precision studies SOFO and LEDI**

| Method | Drug | Conc. (µg/ml) | Intraday Precision | | Intraday Precision | |
|---|---|---|---|---|---|---|
| | | | Mean± SD | %Amt Found | Mean± SD | %Amt Found |
| UV Method | SOFO | 16 | 0.41± 0.00 | 102.00 | 0.21± 0.00 | 101.00 |
| | | 24 | 0.55± 0.01 | 97.79 | 0.29 ±0.00 | 97.25 |
| | | 32 | 0.72 ±0.00 | 101.31 | 0.40± 0.01 | 102.00 |
| | LEDI | 4 | 0.41 ±0.00 | 103.06 | 0.33 ±0.00 | 101.67 |
| | | 6 | 0.61± 0.00 | 102.67 | 0.47 ±0.00 | 100.23 |
| | | 8 | 0.77± 0.00 | 97.38 | 0.62± 0.00 | 101.77 |

***Mean of each 3 reading for UV method**

**Limit of detection (LOD) and Limit of quantification (LOQ)**

LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantify under the stated experimental conditions. LOQ is the lowest concentration of analyte in a sample that can be determined with the acceptable precision and accuracy under stated experimental conditions. The LOD and LOQ of Sofosbuvir was found to be 0.047 µg/ml and 0.142 µg/ml, Ledipasvir were found to be 1.02 µg/ml and 3.11 µg/ml, respectively.

## DISCUSSION

The proposed methods for simultaneous estimation of SOFO and LEDI in tablet dosage forms were found to be simple, accurate, economical and rapid. The method was validated as per the ICH Q2 (R1) guidelines. Standard calibration yielded correlation coefficient ($r^2$) 0.998 for SOFO and 0.997 for LEDI at all the selected wavelengths. The values of % RSD are within the prescribed limit of 2 %, showing high precision of methods and recovery was close to 100% for both drugs. Results of the analysis of pharmaceutical formulations reveal that the proposed method is suitable for their simultaneous determination with virtually no interference of any additive present in pharmaceutical formulations. Hence, the above methods can be applied successfully for simultaneous estimation of SOFO and LEDI in formulations.

## CONCLUSION

The developed UV spectrophotometric method in that linearity, precision, range and robustness were found to be more accurate, precise and reproducible. The methods were found to be simple and time saving. All proposed methods could be applied for routine analysis in quality control laboratories.

## ABBREVIATION USED

**UV:** Ultraviolet; **HPLC:** High Performance Liquid Chromatography; **SOFO:** Sofosbuvir; **LEDI:** Ledipasvir; **LC-MS:** Liquid Chromatography Mass Spectroscopy; **GC:** Gas Chromatography; **ICH:** International Conference on Harmonization; **RSD:** Relative Standard Deviation; **RT:** Retention Time; **SD:** Standard Deviation.

## CONFLICT OF INTEREST

Authors have no conflicts of interest to declare.

## ACKNOWLEDGEMENTS

The authors are thankful to the Management and Principal, Gangamai College of Pharmacy, Nagaon, Dist. Dhule for providing necessary facilities for research work. They are also grateful to R.S.I.T.C Jalgaon, for giving gift samples of pure drugs.

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