New Simple Spectrophotometric Method for Simultaneous Determination of Binary Mixture of Sofusbuvir and Velpatasivir in Bulk and Dosage Form

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**A B S T R A C T**

Two simple accurate reproducible and non-sophisticated Spectrophotometric methods were developed and validated for simultaneous determination of sofosbuvir and velpatasivir without prior separation. The first method was based on employing simultaneous equation method for analysis of both drugs. sofosbuvir and velpatasivir have shown absorbance maxima at 260 and 296 nm in methanol, respectively. Method (II) involves the formation of Q-absorbance equation using the respective absorptivity values at 271nm (isoabsorptive point) and 296 nm (\(\lambda_{\text{max}}\) of VEL). The drugs obey Beer’s Lambert’s law in the concentration range of 1–60µg/mL for both SOF and VEL (for Method I) and in the range of 2–60 µg/mL for SOF and VEL, respectively (for Method II). The accuracy and precision were determined and recovery studies confirmed the accuracy of the developed methods that were carried out following the International Conference on Harmonization (ICH) guidelines. Statistical comparison of the suggested methods with the reported spectrophotometric one using F and t tests showed no significant difference regarding both accuracy and precision.

**Keywords**: Simultaneous equation method, Binary mixture, Q-absorbance method, Velpatasvir, Sofosbuvir, Isosbiestic point.

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1. Introduction

Hepatitis C infection is a major issue concerned to global health. According to the centers for disease control and prevention estimates, of the people infected with liver diseases, about 3 million deaths occur worldwide each year due to hepatitis C virus (HCV) related causes. Despite the rapid development of new therapies, including interferon-free regimens, there remains an unmet medical need for certain groups of patients with hepatitis C virus infection, in particular for those with genotype 2 and 3 and severe liver disease. Epclusa is a combination product containing sofosbuvir 400 mg and velpatasvir 100 mg. Epclusa is a prescription medicine used to treat adults with chronic hepatitis C infection. Sofosbuvir is (S)-isopropyl 2-((S)-((2R,3R,4S)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran-2-yl) methoxy)-(phenoxy) phosphoryl amino)propanoate (figure 1) corresponding to the molecular formula C$_2$H$_2$FNO$_4$P and has a relative molecular mass of 529 g/mol. It is a nucleotide prodrug,[1-3] used for treatment of patients with genotypes 1 to 6 HCV infections with or without use of Ribavirin.[5,6,8] Velpatasvir chemically designated as Methyl ((1R)-2-((2S,4S)-2-(5-((2S,5S)-1-((2S)-2-[(methoxy carbonyl)amino]-3-methylbutanoyl)-5-methylpyrrolidin-2-yl]-1,11-dihydro[2] benzopyran [4’,3’:6,7] naphtho[2,1-d]imidazol-9-yl)-1H-imidazol-2-yl)-4-(methoxy dimethyl) pyrrolidin-1-yl)-2-oxo-1-phenylethyl) carbamate (Figure 2) is a novel HCV nonstructural protein 5A (NS5A)[4] inhibitor that is being developed in combination with sofosbuvir and other direct acting antivirals for the treatment of HCV infection [4,7]. Due to the additive antiviral interaction and lack of cross-resistance observed in vitro between Sofosbuvir and Velpatasvir, the administration of these 2 drugs as a film-coated tablet is expected to provide significant antiviral activity and a favourable resistance profile[9,10,11]. Literature survey revealed that few RP-HPLC methods[13-19] was reported for determination of the binary mixture in tabletdosage form. But so far one spectrophotometric method [20,21] and one bio analytical method[12] has been reported for simultaneous determination of SOF and VEL in combination; hence an attempt has been madeto develop simple, sensitive, rapid, precise, accurate and economic methods to analyze the studied drugs simultaneously by two spectrophotometric methods, simultaneous equation and Qanalysis methods. The proposed methods have been optimizedand validated as per the International Conference on Harmonization (ICH) guidelines and were found to complywith the acceptance criteria.

![Figure 1: Chemical Structure of Sofosbuvir](image1)

2. Experimental

**Apparatus**

**Spectrophotometer:** SYSTRONICS UV-2202 PC, dual beam UV–visible spectrophotometer with two matched 1 cm quartz cells, connected to an HP compatible personal computer (PC) and an HP-600 inkjet printer. Bundled UV–PC personal spectroscopy software version (3.7) was used to process the absorption and the derivative spectra. The spectral band width was 2 nm with wavelength scanning speed of 2800 nm min−1.

**Materials**

**Pure samples:** Sofosbuvir and Velpatasvir were kindly supplied by Hetero drugs pvt ltd Hyderabad. Their purity was found to be 99.84 ± 1.26 and 100.50 ± 0.71, respectively, according to the manufacturer’s direct spectrophotometric method (personal communication).

**Market samples:**

Epclusa tablets- It was labelled to contain 400 and 100 mg Sofosbuvir and Velpatasvir respectively, per tablet.

**Chemicals and reagents:**

Methanol (AR Grade) was purchased from Merck (India) Ltd., Mumbai, India. AR grade chemicals and distilled water were used during experimentation.

**Stock solutions**

Standard stock solutions each containing 1000µg/mL of SOF and VEL were prepared separately in methanol and water 1:1. Working standard solutions of these drugs (100 µg/mL) were obtained by dilution of the respective stock solutions in distilled water.

**Procedure**

**Spectral characteristics and wavelength selection:**

The absorption spectra of 10µg/mL of each of SOF and VEL were recorded over the range 200–350 nm using Methanol: Water (1:1) as blank. The overlay spectra were observed for selection of the suitable wavelengths for each of the developed methods, Fig. 3.

**Method I: simultaneous equation method:**

Appropriate volume, 1 mL of SOF and VEL standard stock solution was transferred to two separate 10 mL volumetric flasks and the volume was adjusted to mark with water to get concentration 10µg/mL, respectively. The solutions were scanned separately in the UV-region i.e. 400-200 nm. From the overlain spectra (Fig. 3) two wavelengths, 260 nm (max of SOF) and 296 nm (max of VEL) were selected for the formation of simultaneous equation. The A(1%, 1 cm) was determined at both the wavelengths selected for each drug. A set of two simultaneous equations was formed as:

![Figure 2: Chemical structure of Velpatasivir](image2)
Under the optimized experimental conditions, LOD was calculated by putting the values of A1 and A2 (58, 290.9) are E (1%, 1 cm) of VEL at 260 and 296 nm. Cx and Cy are concentrations of Sofosbuvir and Velpatasvir in mg/mL in sample solution. The values of Cx and Cy were calculated by putting the values of A1 and A2 to solve the simultaneous Eqs. 1 and 2[22].

Method II (Q-analysis method):
Standard solutions containing 1–10 µg/mL each of SOF and VEL were prepared separately using distilled water. The absorption spectra of the prepared solutions were recorded in the range of 200–350 nm and the absorbance values at 271 nm (iso) and 296 nm (max of VEL) were measured from which the absorptivity values for both drugs at the selected wavelengths were calculated. The method employs Q values and the concentrations of the studied drugs in the prepared solutions were determined by using the following equations:

\[ C_x = \frac{[Q_m - Q_y]}{[Q_x - Q_y]} \cdot \frac{A}{A_x} \]

\[ C_y = \frac{[Q_m - Q_x]}{[Q_y - Q_x]} \cdot \frac{A}{A_y} \]

Where, Cx and Cy are the concentrations of SOF and VEL in µg/mL, respectively; Qm is the absorbance of sample at 271/nm of sample at 296; Qx is the absorptivity of SOF at 296/nm of SOF at 271; Qy is the absorptivity of VEL at 296/nm of VEL at 271; Ax is the absorptivity of SOF at 271; Ay is the absorptivity of VEL at 271; and A is the absorbance of the sample at 271.

Analysis of laboratory prepared mixtures:
Different laboratories prepared mixtures containing different ratios of SOF and VEL were prepared. Zero order absorption spectra of these mixtures were recorded using Methanol:Water (1:1) as a blank and then the absorbance at 260, and 296 (for Method I) were measured, also the absorbance values at 271 and 296 nm (for Method II) were recorded. From the calculated regression equations, concentrations of SOF and VEL in the prepared mixtures were calculated [23].

Analysis of the marketed formulation: Ten EPCLUSA tablets after removing the coating were weighed and crushed to obtain a fine powder. An accurately weighed tablets powder equivalent to 100 mg of tablet was transferred into 100-mL calibrated measuring flask, 10 mL methanol was added. The prepared solution was sonicated for 45 mins; the volume was completed with methanol:water (1:1) and the solution was then filtered. The filtrate was appropriately diluted with methanol and Water.

Validation methods
Recovery studies: To study the accuracy of the proposed methods, recovery studies were carried out by standard addition method at different levels (80%, 100% and 150%). Known amounts of the studied drugs were separately added to the pure-analyzed tablets powder and the percentage recovery was calculated.

Linearity: Under the optimized experimental conditions, the calibration curve for SOF and VEL was constructed by analyzing a series of standard solutions of the drug. The regression equation for the results were derived using least square method.

Precision: The precision was assessed as RSD% at different levels; repeatability was evaluated by the analysis of three different concentrations of pure drugs (2, 6 and 8 µg/mL) for each in triplicates on the same day and intermediate precision by repeating analysis of the same concentrations of each seven times on four consecutive days.

LOD and LOQ: They were calculated from the standard deviation (d) of the response and the slope of the calibration curve (S) in accordance to the following equations: LOD = 3.3 (σ/S) and LOQ = 10(σ/S).

3. Results and Discussions
Development of simple, rapid, sensitive and accurate analytical methods for routine quantitative determination of samples will reduce unnecessary tedious sample preparations cost, materials and laboratories. UV-spectrophotometric analysis of SOF and VEL show strong spectral overlap which interfere with direct spectrophotometric analysis of the studied drugs without derivatization procedure. On the other hand, the simultaneous equation method and Q-analysis methods provide a simple, rapid, convenient and accurate way for simultaneous analysis of SOF and VEL in their combined dosage form without derivatization procedure. The main step in the development and validation of an analytical method is to improve the conditions and parameters which should be followed in the development and validation [25]. Different solvents were studied (methanol, ethanol, acetonitrile, water, 0.1N HCl and 0.1N NaOH) to develop suitable methods of analysis, the criteria employed were the sensitivity of the method, availability and toxicity of the solvent. From a solvent effect studies spectral behaviors of SOF and VEL, Methanol and Water (1:1) was selected as a solvent for the two suggested methods.

Simultaneous equation method:
As the overlay spectrum of SOF and VEL (Fig. 3) shows that there was interference in quantitation of individual drug at their max due to absorption of another drug at that particular wavelength. So, the simultaneous equation
method was developed for estimation of drugs from the pharmaceutical dosage form.

**Q-analysis (graphical absorbance ratio) method:**
This method depends on the property that for the substance that obeys Beer’s Lambert’s law at all wavelengths, the ratio of absorptivity (or absorbance) values at any two wavelengths are constant, independent of the concentration or path length. This ratio is referred as Q-ratio.[26] One of the two selected wavelengths is an isosbortive point and the other is the wavelength of maximum absorption of one of the two components.[26-29]. From the overlain spectra of the two drugs and their mixture, Fig. 3, it is evident that SOF and VEL show isosbortive points at 271 and 246 nm, SOF has max at 260 nm while VEL has max at 296 nm. Using the absorbance values at 271 nm (iso) and 296 nm (max for VEL) gave the best result regarding selectivity. The absorbance values at 271 and 296 nm for SOF in the range of 1–10 µg/mL were obtained similarly for VEL absorbance values in the range of 2–10 µg/mL were measured, absorptivity coefficients were determined for both drugs and the average values were taken. The values and the absorbance ratio were used to develop the following sets of equations from which the concentration of each component in the sample can be calculated.

\[ \text{CSOF} = (Q_m \cdot 1.37/0.157 - 1.37) \cdot A/0.022 \]
\[ \text{CVEL} = (Q_m \cdot 0.157/0.37 - 0.157) \cdot A/0.0217 \]
Where

CSOF is the concentration of SOF in µg/mL; CVEL is the concentration of VEL in µg/mL; Qm is the absorbance of sample at 271/absorbance of sample at 296; and A is the absorbance of the sample at 271. To test the selectivity of developing methods, they were applied for analysis of number of laboratory prepared mixtures containing SOF and VEL in different ratios. The good percentage recoveries and low SD values shown in Table 2, confirming the high selectivity of the suggested methods. The proposed methods have been success fully applied for determination of the studied drugs in bulk powder as well as in their combined dosage form. The results obtained using the suggested methods for analysis of SOF and VEL in EPI USA tablets, Table 2, showed good agreement between the amounts estimated and those claimed by themanufacturer. Moreover, results obtained by the suggested methods showed no significant difference when compared with those obtained by applying the reported spectrophotometric curve [21] as confirmed from F and t values presented in Table 2. The developing methods have advantages over the reported one on being more simple, rapid, economic and can be used for simultaneous determination of the two studied drugs without derivatization or sample pre-treatment.

**Methods validation:** Methods validation has been performed as per the International Conference on Harmonization (ICH) guidelines [30] and USP requirements [31].

**Linearity:**
The linearity of the developed methods was evaluated by analyzing different concentrations of standard solutions of SOF and VEL in triplicates. For Simultaneous method, Beer’s Lambert’s concentration range was found to be 1–10 µg/mL for both SOF and VEL. On the other hand, for Q-analysis the range of SOF was found to be 1–10 µg/mL while for VEL was found to be 2–10 µg/mL. The values of correlation coefficients were close to unity indicating good linearity, the characteristic parameters for the constructed equations are summarized in Table 1.

**Specificity:**
The specificity of the proposed methods was assessed by their application to the analysis of laboratory prepared mixtures containing different ratios of intact SOF and VEL. Satisfactory results were obtained and presented in Table 2, confirming that each of the cited drugs could be successfully determined without interference from the other.

**Accuracy:**
Accuracy was calculated as the percentage recoveries of blind samples of pure SOF and VEL and it indicated the agreement between obtained results and those accepted as true, detailed results are presented in Table 1. Percentage recoveries for SOF and VEL by both the two methods were found to be acceptable, Table 2.

**Precision**
The results of intra-day and inter-day precision confirmed the precision of the proposed methods, Table 1.

**Limits of detection (LOD) and quantitation (LOQ):**
Results presented in Table 1, indicated that the method is sensitive for determination of the studied drugs.

**Comparison with the reported method:**
The results of developed methods were compared with the reported method and expressed in terms of t value and F value (Table 2). The calculated F value was less than the critical value 6.39 for variance at a 0.05%. The calculated t value was also less than the theoretical critical value 2.776 for the two optimized spectroscopic methods. The differences between means were considered insignificant. The comparison with the reported method shows that the developed methods are accurate and precise.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Method I</th>
<th>Method II</th>
</tr>
</thead>
<tbody>
<tr>
<td>max</td>
<td>260</td>
<td>296</td>
</tr>
<tr>
<td>Linearity range</td>
<td>1-60 µg/ml</td>
<td>1-50 µg/ml</td>
</tr>
<tr>
<td>Regression equation</td>
<td>Y=0.03915X+0.002</td>
<td>Y=0.02855X+0.0059</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9996</td>
<td>0.9998</td>
</tr>
<tr>
<td>Slope</td>
<td>0.03915</td>
<td>0.02855</td>
</tr>
<tr>
<td>Limit of detection(µg/ml)</td>
<td>0.119</td>
<td>0.097</td>
</tr>
<tr>
<td>Limit of</td>
<td>0.36</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Table 1: Optical characteristics and validation of proposed method
### Table 2: Determination of studied drugs in Lab prepared mixtures Pharmaceutical preparation by the proposed method and statistical comparison with reported spectrophotometric method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SEM Method</th>
<th>Q Analysis Method</th>
<th>Reported Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOF</td>
<td>VEL</td>
<td>SOF</td>
</tr>
<tr>
<td>Accuracy</td>
<td>101.54±1.06</td>
<td>100.41±1.05</td>
<td>102.58±0.97</td>
</tr>
<tr>
<td>LP Mixtures</td>
<td>102.4±0.376</td>
<td>100.12±1.093</td>
<td>102.32±0.446</td>
</tr>
<tr>
<td>Tablets</td>
<td>96.84±0.77</td>
<td>96.45±0.59</td>
<td>92.75±0.797</td>
</tr>
<tr>
<td>Std addition</td>
<td>101.2±0.58</td>
<td>99.68±0.72</td>
<td>101.59±0.344</td>
</tr>
<tr>
<td>F test(6.388)⁴</td>
<td>1.124</td>
<td>1.000</td>
<td>1.324</td>
</tr>
<tr>
<td>t test(2.306)⁷</td>
<td>1.147</td>
<td>0.642</td>
<td>0.376</td>
</tr>
</tbody>
</table>

⁴ First derivative spectrophotometric determination of SOF at 250 nm and VEL at 260 nm using CH3OH as a solvent.[20]  
⁵ Average of three determinations.  
⁶ Average of six determinations.  
⁷ The values in the parenthesis are the corresponding theoretical values at p = 0.05.

### 4. Conclusion

The developed methods have been successfully applied for simultaneous determination of SOF and VEL in combined sample solution, they were found to be rapid, simple, sensitive and accurate. Once the equations were constructed, analysis required only measuring the absorbance values of the sample solution at the selected wavelengths followed by few simple Calculations. The suggested methods were completely validated showing satisfactory data for all the method validation parameters tested. Recovery studies indicated that practically there was no interference from tablet additives, so these method scan be easily and conveniently adopted for routine quality control analysis of SOF and VEL.

### 5. References


[14] Kalpana N, Shnamukahakumar N.K. Analytical method development and validation for simultaneous estimation of sofosbuvir and...


