Two novel UPLC methods utilizing two different analytical columns and different detection approaches for the simultaneous analysis of velpatasvir and sofosbuvir: application to their co-formulated tablet

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Abstract
In the present study two different RSLC columns, Acclaim RSLC 120 C18, 5.0 µm, 4.6 × 150 mm (column A) and Acclaim RSLC 120 C18, 2.2 µm, 2.1 × 100 mm (Column B) were utilized for the analysis of velpatasvir (VPS) in presence of sofosbuvir (SFV), where due to the encountered fluorescent properties of VPS fluorescent detection at 405 nm after excitation at 340 nm (Method 1) was used for its detection where the non‑fluorescent SFV did not interfere. The same columns were further utilized for the simultaneous determination of SFV and VPS either in bulk form or in their combined tablet, where UV‑spectrophotometric detection at 260 nm was selected for the simultaneous analysis of both drugs (Method 2). A mobile phase consisting of NaH2PO4, pH 2.5 (with phosphoric acid) and acetonitrile in a ratio of 60:40 v/v was used for both methods. The mobile phase was pumped at a flow rate of 1.0 mL/min when using column A and 0.5 mL/min when using column B. The methods showed good linearity over the concentration ranges of 1.0–5.0 and 2.5–10.0 ng/mL for VPS when utilizing Method 1 A and B respectively. Where the linearity concentration range was from 30.0–150.0 to 120–600.0 ng/mL for VPS and SFV respectively when applying Method 2. Both methods 1 and 2 were performed by utilizing the two analytical columns. The different chromatographic parameters as retention time, resolution, number of theoretical plates (N), capacity factor, tailing factor and selectivity were carefully optimized. The results show that comparing the performance of the two utilized columns revealed that shorter column (2.1 mm × 100 mm) with small particle packing was superior to the longer column (4.6 × 150 mm) for the analysis of the studied drugs allowing a reduction of the analysis time by 70% without any detrimental effect on performance. This prompts the decrease of the investigation costs by saving money on organic solvents and expanding the overall number of analyses per day.

Keywords: Velpatasvir, Sofosbuvir, UPLC, Fluorescent detection, UV‑spectrophotometric detection

Introduction
Hepatitis C is an infectious liver disease caused by infection with Hepatitis C Virus (HCV) that is considered a very dangerous disease, influencing about from three to five million people in the United States (US) and about one hundred and seventy million people worldwide. This disease is asymptomatic in its early stages however if it becomes chronic it might prompt risky perilous inconveniences, including liver failure, hepatocellular carcinoma and mortality [1]. Velpatasvir (VPS) is methyl [(2S)-1-[(2S,5S)-2-(9-{2-[(2S,4S)-1-{(2R)-[(methoxycarbonyl) amino]-2-phenylacetyl}-4(methoxymethyl)pyrrolidin-2-yl]-1H-imidazol-4-yl]-1,11 dhydrot][4,3′,6,7]
naphtho[1,2-d]imidazol-2-yl)-5-methylpyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl}carbamate, Fig. 1a.

VPS is a Direct-Acting Antiviral (DAA) medication that plays a significant role in the combination therapy of chronic Hepatitis C. HCV is a solitary stranded RNA virus with nine particular genotypes, where, genotype 1 is the most widely recognized type in the United States, and influencing more than 70% of patients suffering from chronic HCV. Since 2011, the presentation of Direct Acting Antivirals (DAAs, for example, VPS) have fundamentally improved chronic hepatitis C treatment. One of the major advantage of VPS is that it has a noteworthy raised boundary to resistance than its previous generation of NS5A inhibitors, as daclatasvir and ledipasvir, this accounts for its high potency and efficacy as a treatment for chronic Hepatitis C [2]. Sofosbuvir (SFV) (isopropyl (2S)-2-[(2R,3R,4R,5R)-5-(2, dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetra hydrofuran-2-yl] methoxy-phenoxy-phosphoryl] amino] pro-panoate) is a nucleotide analog NS5B polymerase

![Image of structural formulae](image_url)

**Fig. 1** The structural formulae of the studied drugs. **a** Velpatasvir (VPS), **b** sofosbuvir (SFV)
inhibitor. SFV is a prodrug that is mainly used for the treatment of HCV, either alone or in combination with other drugs like, VPS, ribavirin, and ledipasvir [3] (Fig. 1b).

In June 2016, the American Association for the Study of Liver Diseases (AASLD) and the Infectious Diseases Society of America (IDSA) approved VPS and SFV combination (Epclusa) as 1st line therapy for the different six genotypes of Hepatitis C [4].

Since the drugs are recently approved, their literature revealed few analytical methods reported up to date, where, SFV alone was determined by applying chromatographic and spectrophotometric techniques [5, 6]. The forced degradation behavior of SFV was investigated by mean of liquid chromatography-tandem mass spectrometry (LC–MS/MS) [7]. Few UPLC-MS/MS techniques were utilized for the simultaneous analysis of SFV and other antiviral drugs like ribavirin, ledipasvir or in presence of its metabolite [8–10].

Different RP-HPLC methods were reported for the simultaneous determination of SFV and VPS either in bulk, combined tablets or biological fluids [11–14], in addition to two spectrofluorometric methods that were recently reported for the assay of VPS in pharmaceutical tablets and body fluids [15, 16].

The main objective of this work was to develop novel UPLC methods for the simultaneous analysis of VPS and SFV utilizing different analytical columns and different detection approaches.

Experimental

Apparatus

Chromatographic analyses were performed using Thermo Scientific Dionex UltiMate 3000 UHPLC Rapid Separation System (Thermo Fisher Scientific Inc., MA, USA), connected to a quaternary rapid separation pump (LPG-3000RS), Ultimate 3000RS autosampler (WPS-3000), rapid separation diode array detector (DAD-3000RS) and rapid separation fluorescence detector (Dionex Ultimate 3000 RS Fluorescence). Data acquisition, peak integration and calibrations were carried out using UHPLC, CHROMELEON7 software, Dionex, Thermo Fisher Scientific, USA. Mobile phases were filtered using Whatman® Nylon membrane filters 0.2 μm, ø47 mm. The mobile phase was degassed with a sonicator of type GT SONIC QTD-series units with digital timer and heater features, GuangDong GT Ultrasonic Co., Ltd, China. Separation was carried on an Acclaim RSLC columns 120 C18 (120A 4.6 × 150 mm, 5.0μm) and Acclaim RSLC 120 C18 (120A 2.1 × 100 mm, 2.2μm) were used for methods A and B respectively. A mobile phase consisting of NaH2PO4, pH 2.5 (with phosphoric acid, 0.2 M) and acetonitrile in a ratio of 60:40 v/v was used for both methods. The mobile phase was vacuum-membrane filtered through a 0.45 μm Millipore membrane filter and degassed for approximately 10 min before use. The flow rate was 1.0 mL/min when using column, A and 0.5 mL/min when using column B. Columns temperature was maintained at 25 °C. For fluorescence detection of VPS, the detector was set at 340/405 nm (Method 1 A and 1B). While for UV detection of both VPS and SFV the detector was set at 260 nm (Method 2A and 2B). The injection volume was 10 uL.

Laboratory prepared mixture analysis

Stock solution of (SFV and VPS) was prepared at the ratio of (4:1), where, 40 and 10 mg of both SFV and VPS were quantitatively transferred to 100 mL volumetric flask and the volume was adjusted with methanol. Working standard solutions were prepared by suitable dilution of the stock solution with mobile phase. All solutions were stored in the refrigerator to keep their stability.

Materials and reagents

All solvents used in this work were of HPLC grade. Ultrapure water was used for all preparations. VPS (≥98%) was purchased from BioVision, Milpitas Boulevard, Milpitas, CA 95035 USA). SFV (99.98 ± 0.741) was obtained from Cayman chemical company, Ann Arbor, USA) [8]. Acetonitrile and methanol (HPLC grade) were obtained from Merck (Germany). Phosphoric acid, analytical grade Merck (Germany). Sodium dihydrogen phosphate (NaH2PO4) was obtained from central drug house (CDH), New Delhi, India. Phosphoric acid (0.2 mol/L) solution was used to adjust pH to 2.5.

Dosage form

Epclusa® (sofosbuvir 400 mg/velpatasvir 100 mg) tablets was manufactured by Gilead Sciences International, Cambridge, UK.

Standard solutions

Stock solutions of concentration 100.0 μg/mL of VPS and SFV were prepared by dissolving 10 mg of pure drug in 100 mL methanol using an ultrasonic bath. Working standard solutions were prepared by suitable dilution of the stock solutions with mobile phase. All solutions were stored in the refrigerator to keep their stability.
graph construction” section, where, corresponding drug concentrations were calculated from the derived regression equations.

**Calibration graph construction**

A calibration curve was created by accurately measuring volumes of the appropriate drugs working standard solutions delivered into a series of 10 mL volumetric flasks in order to prepare a set of standard solutions in the range specified by the method. The standard solutions were completed to volume with the mobile phase and mixed thoroughly. Aliquots of 10 μL were injected (triplicate) into the columns and eluted with the mobile phase under the optimum chromatographic conditions. The peak area was plotted against the concentration of the drug in ng/mL. Consequently, the corresponding regression equations were derived.

**Procedures for tablets**

A precise weight of the blended content of 10 powdered tablets equal to 10.0 mg of VPS and 40.0 mg of SFV was quantitatively conveyed into a 100 mL volumetric flask and around 30 mL methanol was added. The flask contents were sonicated for 30 min, and made to 100 mL with the same solvent. The solution was filtered through cellulose acetate syringe filter. Working standard solutions were prepared by suitable dilution of the filtered solution with mobile phase.

Analysis of the working standard solution was accomplished via adapting procedures cited under “Calibration graph construction” section, where, the nominal contents of the tablet were calculated from the derived regression equations or the calibration curve.

**Results and discussion**

VPS was found to exhibit an intense fluorescence at 405 nm, after excitation at 340 nm. As a consequence, we aimed to utilize this emission band using UPLC coupled with fluorescence detection, to develop a new method for its analysis in presence of SFV, the method was applied for the analysis of the VPS (pure form) in presence of SFV (Method 1) (Figs. 2 and 3). Moreover, an UPLC with UV detection was utilized for the simultaneous analysis of VPS and SFV in their pure form as well as in their combined tablet (Method 2) (Fig. 4a, b). Both methods 1
and 2 were performed utilizing two different analytical columns.

**Optimization of experimental conditions**

**Choice of appropriate wavelength**

VPS was reported to exhibit a very strong fluorescence permitting very sensitive detection. The optimum excitation and emission wavelengths were determined via preliminary scanning of its fluorescence in the mobile phase, VPS was found to exhibit maximum fluorescence intensity at 405 nm after excitation at 340 nm (Fig. 2).

For simultaneous analysis of VPS and SFV, their $\lambda_{\text{max}}$ were determined through spectrophotometric scan where 260 nm was chosen as optimum wavelength for their simultaneous determination.

**Mobile phase composition**

Several modifications in the mobile phase composition were carried out in a trial to optimize the selectivity, efficiency, and resolution of the chromatographic system. These modifications involved, the pH of the mobile phase, the type and ratio of the organic modifier, column temperature and the flow rate. The results achieved are summarized in Tables 1 and 2.

**pH of the mobile phase**

The influence of the pH change on the different chromatographic parameters studied was investigated via changing the pH of the mobile phase and monitoring the consequence change in parameter.

For both methods pH of 2.5 was the optimum pH resulting in a well-defined peak, optimum resolution of both drugs by Method 2 and shortest analysis time.
Different organic modifiers of concentration 40% (v/v) were utilized in this study. These include acetonitrile, methanol and ethanol. It was found that acetonitrile was the organic modifier of choice for both methods resulting in highest number of theoretical plates, maximum resolution and least tailing factor.

**Type of organic modifier of Conc 40% (v/v)**

Different organic modifiers of concentration 40% (v/v) were utilized in this study. These include acetonitrile, methanol and ethanol. It was found that acetonitrile was the organic modifier of choice for both methods resulting in highest number of theoretical plates, maximum resolution and least tailing factor.

**Concentration of organic modifier**

To study the influence of the concentration of acetonitrile on the proposed analysis methods, its concentration was varied over the range of (40–90%, v/v). As the percentage of acetonitrile increases in the mobile phase a marked peak broadening was noticed, with a concomitant decrease in the number of theoretical plates. Hence, a concentration of 40% acetonitrile was selected as the optimal concentration where it provides an optimum combination of peak symmetry, resolution factor and analysis time (Tables 1, 2).

**Flow rate**

The influence of flow rate on the retention time and peak shape was investigated for both methods with utilization of the two comparative columns.

A flow rate of 1.0 mL/min was optimal for both methods when using column, A, where a flow rate of 0.5 mL/min was optimal for good separation within a reasonable elution time when using column B. This is mainly due to the increased back pressure observed when pumping a mobile phase through columns with small particle size packing.

**The effect of column temperature**

The column temperature was altered through the study to attain the suitable temperature for maximum resolution and optimal peak symmetry. Column temperature was varied over the range (30–60 °C), it was found that room temperature was optimal resulting in highest number of theoretical plates, minimal tailing and best resolution (Tables 1, 2).

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**Table 1 Optimization of the chromatographic conditions for determination of VPS by Method 1**

| Parameter | No. of theoretical plates (N) | Capacity factor (k') | Tailing factor (T_f) |
|-----------|-------------------------------|----------------------|---------------------|
|           | Column (A) | Column (B) | Column (A) | Column (B) | Column (A) | Column (B) |
| Column temperature °C | | | | | | |
| Room temperature | 2900.3 | 406.2 | 1.839 | 2.675 | 0.532 | 0.831 |
| 30 °C | 2941.6 | – | 1.987 | – | 0.660 | – |
| 40 °C | 4547.4 | 1009.7 | 2.183 | 2.038 | 0.536 | 0.900 |
| 50 °C | 5030.4 | 644.4 | 2.348 | 3.179 | 0.517 | 0.877 |
| 60 °C | – | 799.3 | – | 3.454 | – | 0.908 |
| pH of mobile phase | | | | | | |
| 2.5 | 2900.3 | 406.2 | 1.839 | 2.675 | 0.532 | 0.831 |
| 3.3 | 3921.4 | 408.2 | 2.744 | 4.150 | 0.528 | 0.947 |
| 4.0 | 3305.7 | 410.6 | 3.976 | 6.425 | 0.491 | 0.924 |
| Type of organic modifier of Conc 40% (v/v) | | | | | | |
| Acetonitrile | 2720.4 | 378.8 | 1.831 | 2.583 | 0.556 | 0.867 |
| Methanol | 1140.1 | 365.4 | 1.843 | 2.621 | 0.514 | 0.907 |
| Ethanol | 2304.3 | 352.9 | 1.870 | 2.592 | 0.413 | 0.871 |
| Ratio organic modifier: mobile phase (acetonitrile) (v/v) | | | | | | |
| 40:60 | 3145.5 | 372.7 | 1.824 | 2.554 | 0.525 | 0.825 |
| 60:40 | 2720.4 | 378.8 | 1.831 | 2.583 | 0.556 | 0.867 |
| 80:20 | 2386.9 | 314.5 | 1.844 | 2.642 | 0.552 | 0.906 |
| 90:10 | 2210.2 | 316.7 | 1.848 | 2.654 | 0.575 | 0.866 |
| Effect of flow rate (mL/min) | | | | | | |
| 0.3 | – | 452.8 | – | 4.613 | – | 0.768 |
| 0.5 | – | 205.0 | – | 1.433 | – | 0.750 |
| 1.0 | 3551.6 | – | 2.376 | – | 0.571 |
| 1.2 | 2900.3 | – | 1.839 | – | 0.532 | – |

Number of theoretical plates (N) = \( \frac{5.54}{(1 + \frac{\mu}{177})^2} \)

Tailing factor (T_f) = \( \frac{W_{0.5}k'}{\mu} = \frac{(t_R - t_0)}{t_0} \)
Table 2  Optimization of the chromatographic conditions for the determination of SFV by Method 2

| Parameter                          | No. of theoretical plates (N) | Capacity factor (k') | Tailing factor (T_f) |
|------------------------------------|-------------------------------|----------------------|---------------------|
|                                    | Column (A) | Column (B) | Column (A) | Column (B) | Column (A) | Column (B) |
| Column temperature °C              |            |            |            |            |            |            |
| 25 °C                              | 4354.9    | 1014.2    | 1.147     | 4.638     | 0.475     | 0.629     |
| 40 °C                              | 2950.3    | 756.4     | 1.057     | 4.404     | 0.506     | 0.772     |
| 50 °C                              | 2620.8    | 677.9     | 0.989     | 4.208     | 0.471     | 0.73      |
| 65 °C                              | 1895.1    | 468.9     | 0.871     | 3.792     | 0.468     | 0.679     |
| pH of mobile phase                 |            |            |            |            |            |            |
| 2.5                                | 4354.9    | 1014.2    | 1.147     | 4.638     | 0.475     | 0.629     |
| 3.5                                | 3981.2    | 1089      | 1.145     | 4.667     | 0.526     | 0.588     |
| 5                                  | 4617.2    | 1066.9    | 1.145     | 4.667     | 0.455     | 0.572     |
| Type of organic modifier of Conc 40% (v/v) |            |            |            |            |            |            |
| Acetonitrile                       | 4390.4    | 1004.5    | 1.236     | 4.667     | 0.446     | 0.679     |
| Methanol                           | 5073.4    | 1214.5    | 1.179     | 4.738     | 0.474     | 0.664     |
| Ethanol                            | 3342      | 925.4     | 1.175     | 4.708     | 0.481     | 0.65      |
| Ratio organic modifier: mobile phase (Acetonitrile) (v/v) |            |            |            |            |            |            |
| 40:60                              | 4354.9    | 1100.3    | 1.147     | 4.696     | 0.475     | 0.677     |
| 60:40                              | 1925.3    | 1004.5    | 1.173     | 4.667     | 0.453     | 0.679     |
| 80:20                              | 7501.7    | 1648.5    | 1.187     | 4.75      | 0.468     | 0.647     |
| 90:10                              | 8304.6    | 2064.8    | 1.19      | 4.792     | 0.458     | 0.653     |
| Effect of flow rate (mL/min)       |            |            |            |            |            |            |
| 0.3                                | 1041      | 8.196     | 0.816     |            |            |            |
| 0.5                                | 1090.7    | 4.613     | 0.613     |            |            |            |
| 1                                  | 5093.6    | 1.53      | 0.287     |            |            |            |
| 1.2                                | 1117.9    | 1.175     | 0.465     |            |            |            |

Number of theoretical plates (N) = 5.54 \left( \frac{t_R}{W_{0.5}} \right)^2

Tailing factor (T_f) = \frac{W_0.5\cdot k'}{t_R - t_0} / t_0,

Table 3  Analytical performance data for the determination of VPS by Method 1

| Parameter                          | Value |
|------------------------------------|-------|
|                                    | Column (A) | Column (B) |
| Linearity and range (ng/mL)        | 1.0–5.0   | 2.5–10.0   |
| Correlation coefficient (r)        | 1.0      | 0.9999     |
| Slope                              | 20,723.82 | 49,677.50  |
| Intercept                          | -745.284 | -21,790.770|
| S_y, SD of the residuals           | 346.597  | 1808.52    |
| S_ν, SD of the intercept           | 363.51   | 1871.66    |
| S_ν, SD of the slope               | 109.60   | 296.12     |
| SD                                 | 0.89     | 0.97       |
| %RSD\(^a\)                         | 0.888    | 0.97       |
| %Error\(^b\)                       | 0.398    | 0.435      |
| LOD\(^c\)                          | 0.06     | 0.12       |
| LOQ\(^d\)                          | 0.18     | 0.38       |

\(^a\) Percentage relative standard deviation
\(^b\) Percentage relative error
\(^c\) Limit of detection
\(^d\) Limit of quantitation

Method validation

The validity of the proposed UPLC methods was examined in terms of linearity, ranges, limits of detection, limits of quantification, accuracy, precision, robustness, specificity, stability of standard solutions and mobile phase.

**Linearity and range**

Under the above-demonstrated experimental conditions, a linear relationship was obtained by plotting the peak areas against the drugs concentrations. The graphs were found to be rectilinear over the concentration ranges referred to in Tables 3, 4.

Statistical analysis [17] of the data showed high values of the correlation coefficient (r) of the regression equation, minute values of the standard deviation of residuals (Sy/x), of intercept (Sa) and of slope (Sb), and small value of the percentage relative standard deviation and the percentage relative error (Tables 3, 4). These values demonstrated the linearity of the alignment diagrams.
Limits of quantitation and limits of detection

Limits of quantitation (LOQ) and limits of detection (LOD) were evaluated according to ICH Q2R1 recommendations using the following equation [18]:

\[
\text{LOQ} = \frac{10S_a}{b} \quad \text{and} \quad \text{LOD} = \frac{3.3S_a}{b}
\]

where \(S_a\) = standard deviation of the intercept of the calibration curves and \(b\) = slope of the calibration curves.

The values of LOD and LOQ are summarized in Tables 3 and 4.

Accuracy

To demonstrate the accuracy of the proposed techniques, the results of the assay of the studied drugs were contrasted with those of the comparison HPLC method [11]. Statistical analysis of the results using Student’s t test and variance ratio F-test [17] uncovered no huge distinction between the performance of the methods in regard to accuracy and precision, individually (Tables 5, 6).

Precision

The intraday precision was assessed through repeat investigation of various concentrations of the studied drugs in pure form within the explicit working concentration ranges.

Each sample was investigated three consecutive times. Likewise, the interday precision was assessed through triplicate examination of the three specified concentrations on three progressive days. The results for both intraday and interday are summarized in Tables 5 and 6. The relative standard deviations were found to be very deliberate showing sensible repeatability and intermediate precision of the proposed techniques (Tables 7, 8, 9).

Robustness

For the assessment of the techniques robustness, one chromatographic parameter was varied while maintaining all others unaltered. The contemplated variables included; concentration of organic modifier (40%\(\pm\)0.1) and pH of the mobile phase (2.5\(\pm\)0.1). These minor changes did not affect the chromatographic separation or the resolution of the studied drugs from each other.

Specificity

Specificity is the capability to estimate unequivocally the analytes in presence of other components that might be present [18]. Methods specificity was assessed by investigating diverse laboratory prepared mixtures of VPS and SFV at their specified pharmaceutical ratio (Tables 10, 11). It was additionally demonstrated by its capacity to determine VPS and SFV in their pharmaceutical tablets without interference from regular excipients.

Stability of standard solutions and mobile phase

Stock solution stability was studied and evaluated by quantitation of the drugs in comparison to freshly
Table 5 Application of Method 1 for the analysis of VPS in its pure forms

| Studied drug | Proposed method | Comparison method [11] |
|--------------|-----------------|------------------------|
|              | Amount taken (ng/mL) | Amount found (ng/mL) | % Found | % Found |
|              | Column (A) | Column (B) | Column (A) | Column (B) | Column (A) | Column (B) | % Found |
| VPS          | 1.0       | 2.5       | 1.016     | 2.469     | 101.64    | 98.79     | 101.12  |
|              | 2.0       | 3.5       | 1.989     | 3.551     | 99.47     | 101.47    | 102.45  |
|              | 3.0       | 5.0       | 2.987     | 4.980     | 99.57     | 99.60     | 99.78   |
|              | 4.0       | 7.5       | 3.992     | 7.497     | 99.80     | 99.96     | 101.77  |
|              | 5.0       | 10.0      | 5.015     | 10.002    | 100.30    | 100.02    | 99.14   |
| Mean         |           |           | 0.89      | 0.97      | 1.37      |           |        |
| ± SD         |           |           | 0.95      | 1.17      | (2.31)*   |           |        |
| F-test       |           |           | 2.39      | 1.99      | (6.39)*   |           |        |

Each result is the average of three separate determinations

* Figures between parentheses are the tabulated t and F values at P = 0.05 [17]

Table 6 Application of Method 2 for the determination of the studied drugs in their pure form

| Studied drug | Proposed method | Comparison method [11] |
|--------------|-----------------|------------------------|
|              | Amount taken (ng/mL) | Amount found (ng/mL) | % Found | % Found |
|              | Column (A) | Column (B) | Column (A) | Column (B) | Column (A) | Column (B) | % Found |
| SFV          | 120.0     | 120.0     | 121.208    | 120.765    | 101.01    | 100.64    | 100.61  |
|              | 200.0     | 200.0     | 200.205    | 199.979    | 100.10    | 99.99     | 99.71   |
|              | 280.0     | 280.0     | 279.372    | 279.766    | 99.78     | 99.92     | 99.82   |
|              | 400.0     | 400.0     | 399.597    | 400.431    | 99.90     | 100.11    | 99.89   |
|              | 600.0     | 600.0     | 601.597    | 600.989    | 100.27    | 100.16    | 100.06  |
| Mean         |           |           | 100.21     | 100.16     | 100.02    |           |        |
| ± SD         |           |           | 0.48       | 0.28       | 0.354     |           |        |
| t-test       |           |           | 0.72       | 0.72       | (2.31)*   |           |        |
| F-test       |           |           | 1.86       | 1.57       | (6.39)*   |           |        |

VPS

|              | 30.0       | 30.0       | 29.134     | 30.045     | 97.11     | 100.15     | 96.41  |
|              | 50.0       | 50.0       | 51.597     | 49.907     | 103.19    | 99.81      | 98.24  |
|              | 70.0       | 70.0       | 69.821     | 70.056     | 99.74     | 100.08     | 98.10  |
|              | 100.0      | 100.0      | 100.119    | 99.523     | 100.12    | 99.52      | 99.58  |
|              | 150.0      | 150.0      | 150.321    | 149.981    | 100.21    | 99.99      | 99.47  |
| Mean         |           |           | 100.07     | 99.91      | 98.36     |           |        |
| ± SD         |           |           | 2.16       | 0.25       | 1.29      |           |        |
| t-test       |           |           | 2.06       | 0.95       | (2.78)*   |           |        |
| F-test       |           |           | 2.82 (6.39)*| 0.0009 (0.156)*| |        |

Each result is the average of three separate determinations

* Figures between parentheses are the tabulated t and F values at P = 0.05 [17]

prepared standard solutions. No remarkable variation was noticed in the response to standard solutions, compared to freshly prepared standards. Furthermore, the stability of the mobile phase was examined in a similar method. In both methods the results demonstrated that sample solutions and mobile phase applied during the analysis were stable up to 3 days when preserved in the refrigerator at 4 °C.
Table 7 Precision data for the determination of VPS by Method 1

| Parameters | Column (A) | | Column (B) |
|-----------|------------|------------|------------|
| Concentration (ng/mL) | | Concentration (ng/mL) | |
| 1.0 | 3.0 | 5.0 | | 2.5 | 5.0 | 10.0 |
| Intraday | | | | |
| % Found | 96.83 | 98.30 | 99.75 | 94.23 | 93.28 | 99.21 |
| | 97.78 | 98.77 | 99.71 | 92.00 | 91.55 | 98.72 |
| | 94.82 | 97.66 | 99.52 | 93.23 | 96.26 | 98.60 |
| (x) | 96.48 | 98.24 | 99.66 | 93.15 | 93.70 | 98.84 |
| ±SD | 1.51 | 0.56 | 0.12 | 1.12 | 2.38 | 0.32 |
| %RSD | 1.57 | 0.57 | 0.12 | 1.2 | 2.54 | 0.33 |
| %Error | 0.9 | 0.33 | 0.07 | 0.69 | 1.47 | 0.19 |
| Inter-day | | | | | |
| % Found | 98.24 | 98.30 | 99.72 | 92.97 | 96.61 | 99.30 |
| | 99.44 | 96.31 | 100.42 | 100.22 | 99.13 | 100.66 |
| | 99.44 | 96.31 | 100.42 | 93.72 | 99.51 | 99.79 |
| (x) | 99.04 | 96.97 | 100.19 | 95.46 | 98.42 | 99.92 |
| ±SD | 0.69 | 1.15 | 0.40 | 3.99 | 1.58 | 0.69 |
| %RSD | 0.70 | 1.19 | 0.40 | 4.17 | 1.60 | 0.69 |
| %Error | 0.40 | 0.68 | 0.23 | 2.41 | 0.92 | 0.40 |

Each result is the average of three separate determinations

Table 8 Precision data for the determination of VPS by Method 2

| Parameters | Column (A) | | Column (B) |
|-----------|------------|------------|------------|
| Concentration (ng/mL) | | Concentration (ng/mL) | |
| 30 | 70 | 150 | | 30 | 70 | 150 |
| Intraday | | | | | |
| % Found | 95.70 | 99.06 | 99.83 | 101.05 | 99.51 | 99.77 |
| | 93.62 | 97.15 | 99.76 | 92.25 | 96.37 | 97.52 |
| | 100.94 | 100.58 | 99.79 | 95.06 | 97.59 | 99.50 |
| (x) | 96.75 | 98.93 | 99.79 | 96.12 | 97.82 | 98.93 |
| ±SD | 3.77 | 1.72 | 0.04 | 4.50 | 1.58 | 1.23 |
| %RSD | 3.90 | 1.74 | 0.04 | 4.68 | 1.62 | 1.24 |
| %Error | 2.25 | 1.00 | 0.02 | 2.70 | 0.93 | 0.72 |
| Inter-day | | | | | | |
| % Found | 98.69 | 97.19 | 99.92 | 100.19 | 98.41 | 100.21 |
| | 101.55 | 98.21 | 100.13 | 96.79 | 103.68 | 97.25 |
| | 97.43 | 98.37 | 99.87 | 97.74 | 98.49 | 99.77 |
| (x) | 99.22 | 97.92 | 99.97 | 98.24 | 100.19 | 99.80 |
| ±SD | 2.11 | 0.64 | 0.14 | 1.75 | 3.02 | 1.60 |
| %RSD | 2.13 | 0.65 | 0.14 | 1.79 | 3.01 | 1.61 |
| %Error | 1.22 | 0.38 | 0.08 | 1.03 | 1.74 | 0.93 |

Each result is the average of three separate determinations
### Table 9 Precision data for the determination of SFV by Method 2

| Parameters | Column (A) | Column (B) |
|------------|------------|------------|
| Concentration (ng/mL) | | |
| 30 | 70 | 150 |
| 30 | 70 | 150 |

| | | |
|---|---|---|
| Intraday | | |
| % Found | 98.69 | 99.41 | 99.82 |
| 103.37 | 101.32 | 100.28 |
| 100.70 | 99.55 | 100.05 |
| (x) | 100.92 | 100.09 | 100.05 |
| ± SD | 2.35 | 1.07 | 0.23 |
| %RSD | 2.33 | 1.06 | 0.23 |
| %Error | 1.34 | 0.61 | 0.13 |

| | | |
|---|---|---|
| Inter-day | | |
| % Found | 100.34 | 99.79 | 99.86 |
| 100.35 | 99.96 | 100.01 |
| 100.38 | 99.77 | 99.98 |
| (x) | 100.36 | 99.84 | 99.95 |
| ± SD | 0.02 | 0.10 | 0.08 |
| %RSD | 0.02 | 0.10 | 0.08 |
| %Error | 0.01 | 0.06 | 0.05 |

### Table 10 Assay results for the determination of VPS in laboratory prepared mixture with SFV at their pharmaceutical ratio by Method 1

| Combination | Proposed method | Comparison method [11] |
|-------------|----------------|-----------------------|
| SFV/SVF mixture 4:1 (w/w) | | |
| Amount taken (ng/mL) | Amount found (ng/mL) | % Found | % Found |
| Column (A) | Column (B) | Column (A) | Column (B) | Column (A) | Column (B) | |
| 1.0 | 2.5 | 0.989 | 2.495 | 98.93 | 99.78 | 101.45 |
| 2.0 | 3.5 | 1.899 | 3.412 | 94.99 | 97.50 | 100.47 |
| 3.0 | 5.0 | 2.947 | 4.913 | 98.22 | 98.26 | 99.12 |
| 4.0 | 7.5 | 3.933 | 7.356 | 98.34 | 98.09 | | |
| 5.0 | 10.0 | 5.003 | 9.996 | 100.07 | 99.96 | | |

| Mean | 98.11 | 98.72 | 100.35 |
| ± SD | 1.89 | 1.09 | 1.17 |
| t-test | −1.82 | 1.99 | (2.45)* |
| F-test | 2.62 (19.24)* | 1.15 (6.94)* |

Each result is the average of three separate determinations

* The figures between parentheses are the tabulated t and F values at P = 0.05 [17]

### Applications

**Analysis of VPS and SFV in a laboratory prepared mixture of their pharmaceutical ratio**

The reported procedures were effective and applicable for the analysis of VPS in a laboratory prepared mixture with SFV in addition to their simultaneous determination at their pharmaceutical ratio (1:4), as well. The experimental results obtained are expressed in Tables 12 and 13. The concentrations of each compound in the synthetic mixture were evaluated according to the linear regression equations. The results were in good agreement with those reported by the reference method [11].
Table 11 Assay results for the determination of VPS and SFV in their laboratory prepared mixture at their pharmaceutical ratio by Method 2

| Studied drug | Proposed method | Comparison method [11] |
|--------------|----------------|------------------------|
|              | Amount taken (ng/mL) | Amount found (ng/mL) | % Found |
|              | Column (A) | Column (B) | Column (A) | Column (B) | Column (A) | Column (B) |
| SFV          | 120        | 120        | 120.468    | 119.742    | 100.39     | 99.56      | 99.98      |
|              | 200        | 200        | 200.140    | 195.560    | 100.07     | 97.78      | 98.93      |
|              | 280        | 280        | 279.720    | 273.000    | 99.90      | 97.50      | 100.83     |
|              | 400        | 400        | 399.520    | 393.600    | 99.88      | 98.40      |           |
|              | 600        | 600        | 588.120    | 600.420    | 98.02      | 100.07     |           |
| Mean         |             |             |            |            | 99.65      | 98.66      | 100.85     |
| ±SD          |             |             |            |            | 0.93       | 1.12       | 0.95       |
| t-test       |             |             |            |            | 0.38       | 1.61       | (2.45)*    |
| F-test       |             |             |            |            | 1.04       | 1.38       | (6.94)*    |
| VPS          | 30          | 30          | 30.036     | 29.817     | 100.12     | 99.39      | 101.45     |
|              | 50          | 50          | 49.420     | 50.305     | 98.84      | 100.61     | 100.47     |
|              | 70          | 70          | 68.768     | 70.455     | 98.24      | 100.65     | 99.12      |
|              | 100         | 100         | 99.140     | 100.130    | 99.14      | 100.13     |           |
|              | 150         | 150         | 149.910    | 149.100    | 99.94      | 99.40      |           |
| Mean         |             |             |            |            | 99.26      | 100.04     | 100.35     |
| ±SD          |             |             |            |            | 0.78       | 0.62       | 1.17       |
| t-test       |             |             |            |            | 1.61       | 0.50       | (2.45)*    |
| F-test       |             |             |            |            | 2.25       | 3.56       | (6.94)*    |

Each result is the average of three separate determinations

* The figures between parentheses are the tabulated t and F values at P = 0.05 [17]

Table 12 Assay results for the determination of VPS in its co-formulated tablet with SFV by Method 1

| Studied drug | Proposed method | Comparison method [11] |
|--------------|----------------|------------------------|
|              | Amount taken (ng/mL) | Amount found (ng/mL) | % Found |
|              | Column (A) | Column (B) | Column (A) | Column (B) | Column (A) | Column (B) |
| Epclusa® tablet (SFV 400 mg/VPS100 mg) | 1.0        | 2.5        | 0.982      | 2.449      | 98.20      | 97.99      | 102.45     |
|              | 3.0        | 5.0        | 2.995      | 4.827      | 99.84      | 96.53      | 101.78     |
|              | 5.0        | 10.0       | 5.049      | 9.782      | 100.97     | 97.82      | 98.91      |
| Mean         |             |             |            |            | 99.67      | 97.45      | 101.05     |
| ±SD          |             |             |            |            | 1.39       | 0.80       | 1.88       |
| t-test       |             |             |            |            | 2.74       | 3.05       | (2.78)*    |
| F-test       |             |             |            |            | 1.82       | 5.55       | (19.0)*    |

Each result is the average of three separate determinations

* The figures between parentheses are the tabulated t and F values at P = 0.05 [17]

Application of the proposed method for quality control of the studied drugs in commercial dosage forms

The proposed methods were successfully applied for the determination of VPS and SFV in their commercially available co-formulated tablets (Figs. 5, 6). The results depicted in Tables 12 and 13 are consistent with those obtained using the comparison HPLC method [11]. Statistical analysis using Student’s t-test and variance ratio F-test [17] revealed no meaningful variation between the performance of the methods concerning the accuracy and precision, respectively. The favorable percentage recoveries with low standard deviation values emphasized that the proposed methods were convenient...
### Table 13 Assay results for the determination of VPS and SFV in their co-formulated tablet by Method 2

| Studied drug | Proposed method | Amount taken (ng/mL) | Amount found (ng/mL) | % Found | % Found | % Found |
|--------------|-----------------|----------------------|----------------------|---------|---------|---------|
|              |                 | Column (A)           | Column (B)           | Column (A) | Column (B) | Column (A) | Column (B) | Column (A) | Column (B) | Column (A) | Column (B) |
| SFV Epclus® tablet (SFV 400 mg/VPS 100 mg) | 200.0 | 200.0 | 196.900 | 199.520 | 98.45 | 99.76 | 99.98 |
|              | 400.0 | 400.0 | 389.760 | 394.920 | 97.44 | 98.73 | 98.93 |
|              | 600.0 | 600.0 | 596.760 | 589.080 | 99.46 | 98.18 | 100.83 |
| Mean         |      |      |          |          | 98.45 | 98.89 | 100.85 |
| ±SD          |      |      |          |          | 1.01 | 0.80 | 0.95 |
| t-test       |      |      |          |          | 1.83 | 1.42 | (2.78)* |
| F-test       |      |      |          |          | 1.12 | 1.41 | (19)* |
| VPS          | 50.0 | 50.0 | 48.810 | 49.955 | 97.62 | 99.91 | 102.45 |
| Epclus® tablet (SFV 400 mg/VPS 100 mg) | 100.0 | 100.0 | 98.000 | 98.040 | 98.00 | 98.04 | 101.78 |
|              | 150.0 | 150.0 | 148.665 | 148.185 | 99.11 | 98.79 | 98.91 |
| Mean         |      |      |          |          | 98.24 | 98.91 | 101.05 |
| ±SD          |      |      |          |          | 0.77 | 0.94 | 1.88 |
| t-test       |      |      |          |          | 2.39 | 1.76 | (2.78)* |
| F-test       |      |      |          |          | 5.89 | 3.99 | (19.0)* |

Each result is the average of three separate determinations

* The figures between parentheses are the tabulated t and F values at P = 0.05 [17]

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**Fig. 5** Typical chromatogram of VPS in its co-formulated tablet with SFV under the described chromatographic conditions (Method 1 A and B). 

- **a** VPS 2.5 ng/mL utilizing column A.
- **b** VPS 1.0 ng/mL utilizing column B.
for the routine determination of the studied compounds in their commercial dosage form.

**Comparison of the two proposed methods**
The present work describes two UPLC methods (1 and 2) with the utilization of two analytical columns (A and B) for the analysis of two antiviral drugs namely VPS and SFV.

Method 1 can detect only VPS without any interference from SFV, the method is highly sensitive when compared to Method 2 and far more selective. In addition, Method 1 A is more sensitive that Method 1 B, however Method 1 B provides shorter analysis time.

Method 2, has the advantage of being able to determine both drugs at the same time, the method is more sensitive when compared to previously reported ones, and provide short analysis time, where Method 2 B can resolve both drugs in less than 1.5 min. Both methods can be applied for quality control analysis of both drugs.

**Conclusion**
The present work represented two convenient UPLC methods for the determination of VPS and SFV. The proposed UPLC approaches have been fully validated and demonstrated accurate assay methods for the determination of VPS and SFV with enhanced sensitivity and specificity. The good validation criteria of the proposed methods allow their application in quality control laboratories.

**Abbreviations**
VPS: velpatasvir; SFV: sofosbuvir; HCV: Hepatitis C Virus; US: United States; DAAs: Direct Acting Antivirals; AASLD: American Association for the Study of Liver Diseases; IDSA: Infectious Diseases Society of America; LC–MS/MS: liquid chromatography–tandem mass spectrometry; UPLC–MS/MS: Ultra performance liquid chromatography–tandem mass spectrometry; RP-HPLC: reversed phase-high performance liquid chromatography; RSLC: rapid separation liquid chromatography; μL: micro liter; μg/mL: microgram per milliliter; v/v: volume per volume; mL/min: milliliter per minute; LOQ: limits of quantitation; LOD: limits of detection; ICH: The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; N: no. of theoretical plates; k′: capacity factor; Tf: tailing factor; Sg: standard deviation of the
intercept; $S_p$: standard deviation of the slope; $SD$: standard deviation; $%RSD$: percentage relative standard deviation; $%Error$: percentage relative error.

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Authors’ contributions
FB provided the authentic drugs and dosage forms, proposed the subject, participated in revision of the manuscript. MM designed the assay, conducted its validation, analysis of the samples, participated in the results. RE, participated in the study design, assay design, literature review, discussion and participated in manuscript preparation, results and discussion. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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