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Rapid and validated UHPLC method for simultaneous determination of sofosbuvir, ledipasvir and paracetamol as commonly repurposed drugs for COVID-19 treatment: application in spiked human plasma

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Abstract

Background Sofosbuvir/ledipasvir (SOF/LDV), a combination of antiviral drugs, has been recently repurposed for COVID-19 management, according to Food and Drug Administration approval. Paracetamol (PAR) identified as a first-line antipyretic for COVID-19 symptoms' management. The use of these three drugs together has significantly influenced the management of COVID-19 by providing symptomatic relief via inhibiting viral activity. A validated ultra-high performance liquid chromatographic (UHPLC) method has been introduced for the quantification of these repurposed drugs in COVID-19 treatment. This novel chromatographic method allows the simultaneous detection of SOF, LDV, and PAR in bulk. Additionally, the method has been applied to determine the levels of SOF and LDV in human plasma samples with PAR used as an internal standard.

Results A new UHPLC method was developed, using a mobile phase with a combination of acetonitrile and 0.1% orthophosphoric acid in a proportion of 42:58 (*v/v*).Flow rate was set at 0.4 ml/min, and UV detection was adjusted at 254 nm. The concentration of SOF, LDV, and PAR were measured by their corresponding peak areas, and showed linear relationships between concentration and peak area within the ranges of (5–60) µg/ml for SOF, (2–22) µg/ml for LDV, and (1–22) µg/ml for PAR. The presented UHPLC method was used to quantify the amounts of SOF, LDV, and PAR in both bulk samples and human plasma samples being spiked with the mentioned analytes. The elution process was completed within 4 min, with retention times of 3.28 min for SOF, 2.28 min for LDV, and 1.70 min for PAR. The method showed high separation selectivity, with an injection volume of 1µl. The precision, accuracy and repeatability of the method were found to be within acceptable limits.

Conclusion The recently developed method has been successfully validated in accordance with the guidelines set by the International Council for Harmonization (ICH). This validation process ensures that the method is suitable for routine quality control analysis, making it convenient for regular use.

Keywords UHPLC, Sofosbuvir, Ledipasvir, Paracetamol, COVID-19, Human plasma

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Background

In December 2019, the Chinese government informed the World Health Organization (WHO) about the occurrence of pneumonia cases in hospitalized patients with an unknown cause. Subsequently, these patients were identified as having COVID-19, caused by the SARS-CoV-2 virus. The WHO declared the outbreak of this coronavirus illness as a global pandemic in March 2020. By the end of June 2020, the total number of reported cases worldwide had exceeded 10 million, with a significant number of deaths [1].

In mild cases, individuals experienced symptoms such as fatigue, fever, and dry cough. However, severe infections led to the failure of the respiratory and renal systems [2]. To manage the emerging COVID-19, numerous studies have been conducted internationally, and several existing medications have been repurposed [3]. Some of these medications include compounds containing heterocyclic structures, which are widely used in the pharmaceutical industry [4]. One such medication is the combination of SOF/LDV, which has been repurposed for the treatment of COVID-19 [5, 6]. SOF/LDV is an FDA-approved combination used for treatment of hepatitis C virus (HCV) infection [7]. SOF works by suppressing the non structural protein 5B—RNA dependent RNA polymerase (NS5B–RdRp) enzyme, which is necessary for hepatitis C virus replication. LDV, on the other side, inhibits the non structural protein 5A(NS5A), a crucial protein required for the function of RdRp [8].

Many researches have been directed for the discovery of drugs capable of reversing the COVID-19 most severe and potentially fatal consequences, particularly hyper coagulation and cytokine storm [9]. Ibuprofen is a popular over-the-counter pain reliever. Recent research, however, have raised concerns regarding its possible hazardous impact with corona virus disease 2019, after French authorities announced in March 2020 the risk of negative effects of ibuprofen in COVID-19 patients via Angiotensin-converting enzyme 2(ACE2) regulation. As a result, PAR is preferred over ibuprofen for the treatment of COVID-19 symptoms [10, 11]. PAR, also known as N-(4-Hydroxyphenyl) acetamide exhibits antipyretic and analgesics actions and was recently identified as the first-line antipyretic in COVID-19 symptomatic management [12].

Sofosbuvir(SOF) also known as (S)-isopropyl-2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydro-pyrimidin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyl-tetrahydro-furan-2-yl)methoxy-phenoxy-phosphoryl) amino) propanoate, is a solid substance, off-white in color. It is non-hygroscopic crystals (Fig. 1a). Ledipasvir (LDV) which is methyl [(2S)-1-{(6S)-6-[5-(9,9-difluoro-7-{2-[(1R,3S,4S)-2-{(2S)-2-[(methoxycarbonyl) amino]-3-methylbutanoyl}-2azabicyclo[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] carbamatepropan-2-one (1:1), is another crystalline substance. It is slightly hygroscopic and forms crystals (Fig. 1b). Paracetamol (PAR),*N*-(4-hydroxyphenyl) acetamide, appears as white solid *crystals* [13] (Fig. 1c).

Upon literature review, many chromatographic techniques, including LC–MS/MS, were known to be used

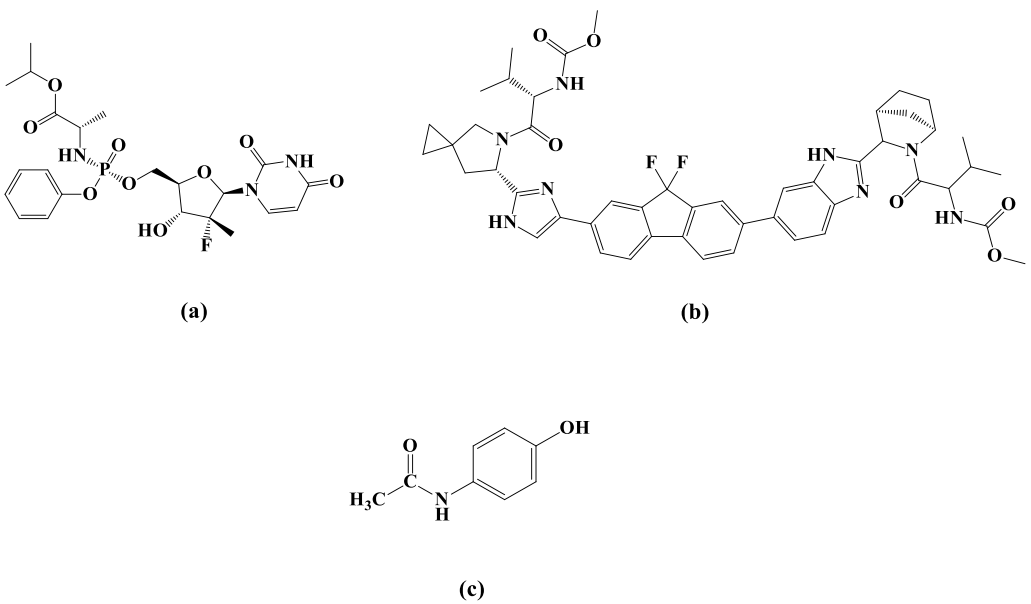

Fig. 1 Chemical structures of **a** SOF, **b** LDV and **c** PAR drugs

for the determination of SOF/LDV in combination [14–16], and RP-HPLC–DAD [17]. Also, dissolution studies applying RP-HPLC were performed [18–20]. On the other hand, several chromatographic techniques for determination of PAR were conducted, including TLC densitometry [21–23] and HPLC approaches [24–26]. Hereby, in this study the pharmacological relevance of combining SOF and LDV with PAR for the treatment of COVID-19 was the urge for the development of convenient method for the analysis of the repurposed drugs.

Material and methods

Instrumentation

Agilent technology 1290 infinity system was utilized for chromatographic separation. This system consisted of various components including a UV Diode array detector (G4212A), a Quaternary pump (G4204A), a thermostat (G330B), a thermostated column compartment (G1316C), an auto injector sampler (G4226A), and Open LAB ChemStation C.01.05 software (USA) for data analysis. For sonication purposes, a Branson Model 3510 Ultrasonic Cleaner from the UK was employed. A high-speed and refrigerated centrifuge (centrifuge sigma 3-30k, Germany) was used for centrifugation. An analytical balance (Italy, Sartorius CPA225D) was utilized for weighing samples accurately. A pH meter instrument (Jenway 3505, UK) was utilized for determining solutions pH. To obtain deionized water, pure lab flex (FLC00006641) was utilized in the laboratory.

Reagents and chemicals

Authentic standards of SOF & LDV (purity 99.5%) were supplied by Optimus in India, and PAR (purity 99.9%) was purchased from EL-Rewad Industrial Pharmaceutical Company (RPIC) in Cairo, Egypt. Methanol, acetonitrile and orthophosphoric acid, all HPLC grade, were obtained from (Sigma-Aldrich, Germany). Shabrawishi Blood Bank, Eldokki, Cairo, Egypt, provided fresh frozen plasma.

Standard and working solutions

Standard stock solutions were prepared by accurately weighing and transferring 0.1 g of SOF, LDV, and PAR to separate 100 ml volumetric flasks and completing the volume with methanol. Stock solutions of 1000 µg/ml concentration were separately produced. UHPLC working solutions(100 µg/ml),were developed by individual transfer of 10 ml of SOF, LDV and PAR standard stock solutions (1000 µg/ml) to 100 ml volumetric flasks and completing the volume with methanol.

Procedures and chromatographic conditions

Various concentrations of SOF, LDV, and PAR, ranging from 5 to 60 µg/ml, 2 to 22 µg/ml, and 1 to 22 µg/ml), respectively, were prepared by transferring different volumes from a working solution of 100 µg/ml into 10 ml volumetric flasks and then completed with methanol. An auto sampler was utilized to inject 1 µl of each sample. To achieve chromatographic separation, an Agilent Infinity Lab 101 Poroshell 120 EC-C18 (3×150 mm 1.9-Micron) column from the USA was employed. The mobile phase consisted of a mixture of acetonitrile and 0.1% orthophosphoric acid in a ratio of 42:58 (v/v), with a flow rate of 0.4 ml/min. UV detection was set at 254 nm using a diode array detector (DAD).

Drug spiked plasma method

Six non-zero drug spiked human plasma calibration standards were prepared with concentration range of 5–35 µg/ml and 4–20 µg/ml for SOF and LDV, respectively. Preparation was completed by adding 50 µl of known working solution of SOF(50–350 µg/ml) and 50 µl of known working solution of LDV (40–200 µg/ml) to 350 µl of drug free human plasma. PAR was used as internal standard by adding 50 µl of 100 µg/ml PAR working solution.

For drug extraction, 500 µl of all drugs spiked calibration plasma standards were mixed with 500 µl of acetonitrile for protein precipitation. The solutions were then vortexed for 10 min then centrifuged at 3000 rpm for 15 min and supernatants were transferred to vials for UHPLC analysis.

Results

Linearity

Linear correlations were established while plotting the peak area against the concentrations of each of: SOF, LDV, and PAR within their concentration ranges of 5–60.0 µg/ml, 2–22 µg/ml, and 1–22 µg/ml, respectively. The obtained results are presented in Table 1 and Fig. 2. It's worth noting that oral doses of the mentioned drugs was reported to show maximum plasma concentrations (C_{max}) of 567 ng/ml and 323 ng/ml for sofosbuvir and ledipasvir, respectively. While after oral administration of acetaminophen, $C_{max}$is 12.3 µg/ml.

Regarding drug spiked human plasma, linear relationships were observed by plotting the ratio of the peak area for each analyte (SOF and LDV) to the peak area of the PAR internal standard (10 µg/ml) against the concentrations of SOF (ranging from 5 to 35 µg/ml) and LDV (ranging from 4 to 20 µg/ml). These findings are depicted in Fig. 3.

Table 1 Validation parameters of the UHPLC method for quantification of SOF, LDV and PAR in bulk

Parameter	UHPLC		
	SOF	LDV	PAR
Range(µg/ml)	5–60	2–22	1–22
Slope	13.39	13.99	61.25
Intercept	− 2.783	− 18.41	58.26
Correlation coefficient(r)	0.9993	0.9998	0.9998
Accuracy (mean ± SD)	99.99 ± 0.554	99.11 ± 0.197	100.362 ± 0.535
Precision (RSD %)			
Intra-day*	0.884	0.938	0.702
Inter-day*	0.776	0.403	0.355
LOD (µg/ml)**	0.153	0.074	0.015
LOQ(µg/ml)***	0.464	0.225	0.045

*Relative standard deviation (n = 3), average of three different concentrations of SOF, LDV and PAR

Limit of detection and* limit of quantitation are determined via calculations

Accuracy

According to ICH recommendations [27], proposed methods' accuracy was assessed by analyzing different concentrations of SOF (15, 25and 40 µg/ml), LDV (8, 15and 18 µg/ml) and PAR (2, 6and18µg/ml). Standard deviation and mean recoveries were determined to be around acceptable parameters, with high accuracy (Table 2). For drug spiked human plasma method, different concentrations of drug spiked human plasma standard SOF (8, 18 and 25 µg/ml) and LDV (7, 14 and 18 µg/ml) were studied (Table 3).

Precision

Precision of the suggested method was valid for both bulk and drug spiked human plasma samples, considering intra-day and inter-day variations. In the case of bulk analysis, intra-day precision was assessed by analyzing three different concentrations of SOF (10, 30, and 60 µg/ml), LDV (5, 10, and 14 µg/ml), and PAR (4, 10, and 22 µg/ml) using the UHPLC method on the same day. Similarly, the same concentrations were analyzed on three different days to evaluate inter-daily precision, as shown in (Table 1).

For the drug spiked human plasma analysis, the precision of the suggested method was examined intra-daily and inter-daily using three different concentrations (low, medium, and high). Intra-day precision was evaluated by analyzing three different concentrations of drug spiked plasma, including SOF (10, 20, and 35 µg/ml) and LDV (6, 10, and 20 µg/ml), on the same day using the UHPLC method with PAR as the internal standard (at a concentration of 10 µg/ml). The same concentrations were analyzed on three different days to assess inter-daily precision.

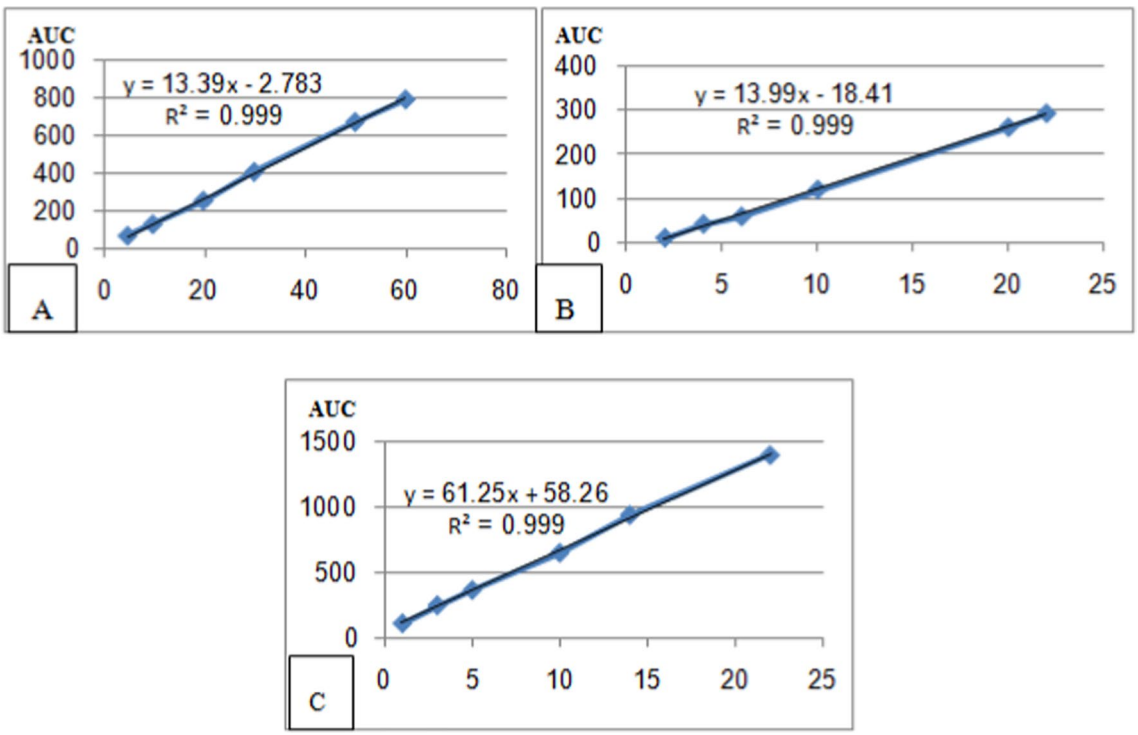

Fig. 2 Linearity curves with regression equations in pure bulk. **A** SOF (5–60.0 µg/mL), **B** LDV (2–22 µg/mL) and **C** PAR (1–22 µg/mL)

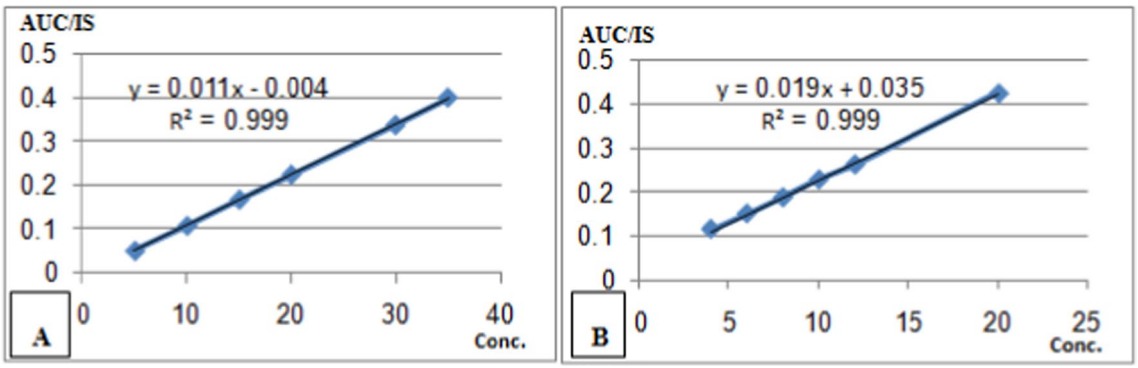

Fig. 3 Linearity curves with regression equations in drug spiked plasma. **A** SOF (5–35 µg/ml) and **B** LDV (4–20 µg/ml) in presence of PAR as internal standard (10 µg/ml)

Table 2 Accuracy results for UHPLC method for the quantification of SOF, LDV and PAR in bulk

SOF			LDV			PAR		
Taken µg/mL	Found µg/mL	Recovery, %[a]	Taken µg/mL	Found µg/mL	Recovery, %[a]	Taken µg/mL	Found µg/mL	Recovery, %[a]
15	15.26	100.51	5	7.91	98.91	2	1.99	99.77
25	24.85	99.41	15	14.89	99.31	6	6.04	100.81
40	40.77	100.07	18	17.842	99.12	18	18.09	100.50
	Mean ± SD			Mean ± SD			Mean ± SD	
	99.99 ± 0.554			99.11 ± 0.197			100.36 ± 0.535	

[a] Average of three determinations

Table 3 Accuracy results for UHPLC method for the quantification of SOF and LDV in spiked human plasma

SOF			LDV		
Taken µg/mL	Found µg/mL	Recovery, %[a]	Taken µg/mL	Found µg/Ml	Recovery, %[a]
8	7.77	97.19	7	6.88	98.32
18	17.77	98.75	14	13.38	95.58
25	24.87	99.49	18	17.29	96.05
	Mean ± SD			Mean ± SD	
	98.48 ± 1.174			96.65 ± 1.465	

[a] Average of three determinations

Specificity

To evaluate the specificity of the proposed techniques, laboratory-made combinations of SOF, LDV, and PAR were prepared at different concentrations and ratios. These combinations were tested to ensure that there was no interference observed in the presence of each other, as depicted in Fig. 4. Additionally, the techniques were assessed for any interference with plasma content, as shown in Fig. 5. The results showed no significant interferences, confirming the specificity of the method.

Furthermore, the mean recoveries obtained from the analysis were found to be acceptable, indicating the accuracy of the suggested method. Additionally, the method demonstrated good resolution, as demonstrated in Table 4.

Robustness

Robustness of the suggested UHPLC method was assessed by examining the impact of small variations in flow rate (0.35 and 0.45 ml/min), the ratio of the mobile phase (40:60 and 44:56 *v/v* acetonitrile: 0.1 orthophosphoric acid), and temperature (30 ± 2 °C). These variations were evaluated to determine their effect on the peak areas. The results obtained demonstrated that the peak

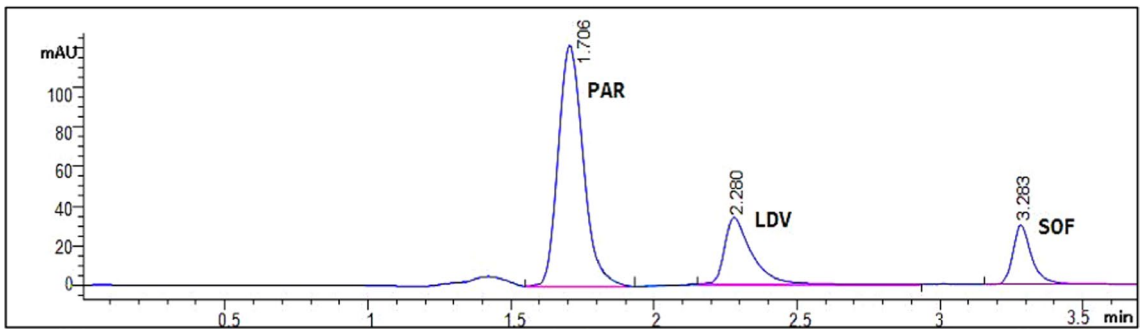

Fig. 4 UHPLC chromatogram of standard solutions of: SOF 10 µg/mL, R_t = 3.28 min, LDV 10 µg/mL, R_t = 2.28 min and PAR 10 µg/mL, R_t = 1.70 min

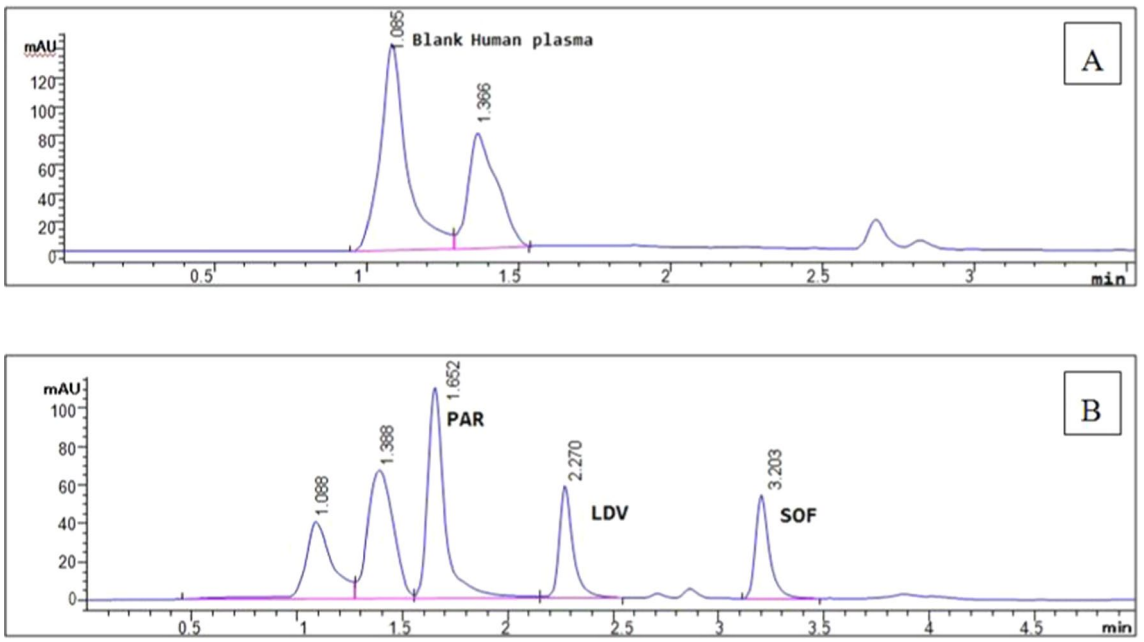

Fig. 5 **A** Chromatogram of blank human plasma showed no drug peak interference. **B** UHPLC chromatogram of analytes in drug spiked human plasma: SOF (35 µg/mL; R_t = 3.2 min), LDV (20 µg/mL; R_t = 2.27 min) using PAR (10 µg/mL; R_t = 1.66 min) as internal standard

Table 4 Quantification of SOF, LDV and PAR in the laboratory prepared mixtures by UHPLC method

Binary mixture SOF: LDV:PAR ratios	%Recovery[a]		
	SOF	LDV	PAR
4: 0.9:5	99.61%	99.86%	100.52%
2: 1:1	98.99%	100.00%	98.68%
1: 2:1	100.31%	98.78%	99.26%
Mean ± SD	99.64 ± 0.65	99.55 ± 0.66	99.48 ± 0.93

[a] Average of three determinations

Table 5 Robustness study of the developed UHPLC method for quantification of SOF, LDV and PAR

UHPLC parameters	SOF	LDV	PAR
	%RSD of peak areas	%RSD of peak areas	%RSD of peak areas
Flow rate [a]	0.645	1.149	0.529
Mobile phase ratio[b]	0.546	0.563	0.793
Temperature[c]	0.537	0.581	0.264

[a] 0.35, 0.45 ml min^{-1} for SOF, LDV and PAR

[b] Ratio of mobile phase (40/60 v/v), (44/56 v/v) (acetonitrile/0.1 opa v/v)

[c] Temperature (30 ± 2 °C)

Table 6 System suitability parameters for quantification of SOF, LDV and PAR

Parameter	Value			Reference value
	SOF	LDV	PAR	
Retention time(min)	3.2	2.3	1.7	
Capacity factor (k')	9.65	7.98	5.55	1–10
Selectivity (α)	1.46	1.44	1.26	$\alpha > 1$
Resolution (Rs)	6.51	3.68	1.5	$R \geq 1.5$
Tailing factor(T)	1.356	1.875	1.184	1–2
Number of Theoretical plates	13,314.2	2622.07	1793.7	Increase with efficiency of separation
HETP*	0.01	0.05	0.08	The smaller the value the higher the column efficiency

*Height equivalent to theoretical plates = column length/Number of Theoretical plates

areas exhibited low %RSD (Relative standard deviation) values, indicating the robustness of the method. These findings are presented in Table 5.

System suitability

The system suitability parameters, such as retention time (min), capacity factor (k'), selectivity (α), resolution (Rs), tailing factor (T), number of theoretical plates (N), and height equivalent to theoretical plates (HETP), were examined and assessed in accordance with the guidelines of US Pharmacopeia [28] (Table 6).

Discussion

Extensive experimentation was conducted to determine the optimal chromatographic conditions for the separation of the analyte mixture. Various parameters, such as column type, mobile phase polarity, pH, and organic solvent ratio, were investigated.

Initially, using a ZORBAX CN column (4.6×250 mm, 5 µm) with a mobile phase consisting of acetonitrile and phosphate buffer in a 50:50 (*v/v*) ratio, adjusted to a pH of 5.8 using orthophosphoric acid, failed to achieve satisfactory separation. This resulted in overlapping peaks. Even after trying different mobile phase ratios, separation could not be achieved. However, when the column was switched to a Eurospher 100–5 C18 column (250×4.6 mm) and a mobile phase ratio of 60:40 (*v/v)* was employed, separation was achieved. However, a forked peak for PAR was observed.

Finally, the best separation results were obtained when an Agilent Infinity lab Poroshell 120 EC-C18 column (3×150 mm, 1.9 µm) was used applying a mobile phase consisting of acetonitrile and 0.1% orthophosphoric acid in a 40:60 (*v/v*) ratio. This system yielded sharp peaks for all the analyzed drugs. To further enhance peak sharpness and reduce retention time, the mobile phase ratio was adjusted to 42:58 (*v/v*) of acetonitrile and 0.1% orthophosphoric acid.

Conclusions

The suggested chromatographic method was utilized for the simultaneous quantification of SOF, LDV, and PAR, which are commonly used together as a repurposed combination for COVID-19 management. The method has been demonstrated to be accurate, precise, specific, and robust. It also features a short run time, ensuring its economic, simple, and fast operation. Consequently, the method can be effectively employed for routine quality control analysis in the pharmaceutical industry. Moreover, the proposed method was successfully applied to drug spiked human plasma analysis and underwent validation in accordance with the guidelines set by the ICH of technical requirements for pharmaceuticals for human use.

Abbreviations

UHPLC	Ultra-high performance liquid chromatography
SOF	Sofosbuvir
LDV	Ledipasvir
FDA	Food and Drug Administration
PAR	Paracetamol
ICH	International Conference on Harmonization
WHO	Food and Drug Administration
HCV	Hepatitis C virus
NS5B	Nonstructural protein 5B
RdRp	RNA dependent RNA polymerase
NS5A	Nonstructural protein 5A
ACE2	Angiotensin-converting enzyme 2

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Author contributions
SG: Conceptualization, Methodology, Analysis, Validation, Visualization, Writing original draft. GGM: Supervision, Visualization & editing. SAS: Supervision, Visualization & editing. MIE: Methodology, Supervision, Visualization, & editing. AAM: Methodology, Validation, Supervision, Visualization, & editing.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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

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