

RESEARCH ARTICLE**DEVELOPMENT AND VALIDATION OF A NEW RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION AND QUANTIFICATION OF SOFOSBUVIR AND DACLATASVIR**

Syed Hassan Murtaza¹, Ahmed Umer Sohaib^{1*}, Tassawer-e-Meran², Sonam Anis³, Najm ul Hassan Khan¹, Kishwar Sultana¹

¹Faculty of Pharmacy, The University of Lahore, Lahore Pakistan. ²Yashfeen College of Pharmacy, Lahore, Pakistan. ³University College of Pharmacy, University of the Punjab, Lahore, Pakistan

*Corresponding author's email: iamsohaib22@gmail.com

DOI: <https://doi.org/10.56536/ijpihs.v5i1.118>

Submitted on: 11-09-2023

Revised on: 10-01-2024

Published on: 01-03-2024

ABSTRACT

Background: Sofosbuvir (SOF) and daclatasvir (DCV) are usually prescribed in combination for the treatment of hepatitis C worldwide. **Objectives:** In the present study a novel, simple, rapid, and cost-effective reversed-phase high performance liquid chromatography (HPLC) method was developed, optimised, and validated for simultaneous analysis of sofosbuvir and daclatasvir in human plasma as well as in solid dosage forms. **Methodology:** The chromatographic analyses were carried out using Shimadzu HPLC gradient system (LC20), while the other HPLC system used for the peak purity and repeated validation studies was Agilent 1260 Infinity fitted with diode array detector. The column used was C18 (150 × 4.6 mm; 5 µm), from Agela Technologies. The mobile phase consisted of acetonitrile and 0.02 M potassium Hexafluorophosphate buffer (42:58, v/v) at pH 2.7, pumped at the rate of 1.3 mL min⁻¹. The limit of detection and limit of quantification were determined using dilution method (RSD/m formula). **Results:** SOF and DCV combination therapy is significant for the treatment of hepatitis C. Development of this RP-HPLC method for the simultaneous determination and quantification of SOF and DCV is a part of an extensive project of novel formulation development (fixed dose combination tablets of SOF and DCV). This method is successfully applied for raw materials as well as in combination tablets. This method may also be applied for the quantification of sofosbuvir and daclatasvir in blood samples of patients taking these two antivirals for hepatitis C treatment. **Conclusion:** The optimized method was successfully applied for the determination of sofosbuvir and daclatasvir in single run without any interference. Hence, the developed method is quite appropriate for the simultaneous determination of SOF and DCV in solid dosage form as well as in human plasma for clinical research studies.

Keywords: Daclatasvir, Optimization, RP-HPLC, Sofosbuvir, Validation

INTRODUCTION

Method development for any pharmaceutical dosage form is a basic step in the discovery of new products. Scientists have focused their attention in developing new/improved dosage forms which may be effective in reducing the duration of treatment, increase bioavailability, cost-effectiveness and enhanced efficacy (1).

In the recent past, many improvements have been made in the treatment of hepatitis C virus (HCV) infection with the development of novel and more potent agents. Previously, the recommended treatment for HCV was PEGylated interferon injections on a week basis with weight-based ribavirin, both for four months, providing a sustained virological response (SVR) rate of 47.5% on average. The very first generation protease inhibitor telaprevir (Incivek, Vertex Pharmaceuticals) appeared in the market in 2011, followed by introduction of boceprevir but these were withdrawn from the market because of introduction of more effective and direct acting drugs (2). The collective use of one of these along with ribavirin and PEGylated interferon alfa-2a (Pegasys, Genentech) improved the SVR rates in patients with HCV genotype 1 up-to 71%. Simeprevir a protease inhibitor of second generation followed this. In 2013 RNA polymerase, sofosbuvir (SOF), an inhibitor of 5B nonstructural protein was approved. On October 10, a combination ledipasvir plus sofosbuvir was approved for genotype 1 hepatitis C by the Food and Drug Administration (FDA) (3).

Academic and industrial research groups efforts and improvements in the HCV life cycle understanding, several successful direct-acting antivirals are approved already or are in last stages of clinical development. HCV causing hepatitis requires multiple regimens to treat this disease (4). The regimens may include any antiviral agent which can inhibit HCV non-structural protein NS5A which is essential protein for HCV replication and nucleotide analog which inhibits the RNA polymerase that is used by the RNA of hepatitis C virus for replication. Both these directly acting antiviral drugs give synergistic effect against hepatitis C (5).

SOF (pyrimidine nucleotide analog) is a new drug candidate for effective treatment of hepatitis C. It is a highly effective inhibitor of the RNA polymerase (NS5B) that is used by the RNA for replication. A very potent response is observed by the Sofosbuvir against HCV with PEG-INF along with other drugs. SOF is having exceptional interest by the scientists due to its oral administration, very few side effects, large barrier to resistance and potency, among all other antivirals. Several clinical trials have proven the high efficacy of SOF in patients with HCV along with its safety. The administration of sofosbuvir for the period of 3 to 6 months with 400 mg dosage per day has given potent results in association with ribavirin and PEG-INF. The combination of sofosbuvir with many directly acting antivirals also have proven promising results (6).

Sofosbuvir is used with many drugs particularly daclatasvir (DCV) having synergistic effect to SOF. A combination of Once daily SOF/ledipasvir is used with ribavirin. Sustained virologic response is obtained in HCV patients using either of the two NS5A inhibitor analogues along with SOF and ribavirin for a treatment duration of 3 months. The efficacy of SOF along with ledipasvir against HCV 1 genotype is also established by another study (7). DCV is a unique, important, and attractive candidate for HCV patient's treatment having chronic infection. A number of inhibitor properties have been observed in many studies by this drug (8).

During the hepatitis C viral response analysis, viral growth has been effectively blocked by SOF and GS-0938 administration in infected cells. Both drugs were observed to have equal and synergistic effect in reducing viral load in all subjects by up-to the average of 99.96% (9). This study revealed that this effective response was not achieved until 48 hours of administration of SOF and GS-0938. As a result of first phase virus decline a fast decline in virus load was observed in second phase at an average of 0.35/day (10). The success of SOF along with SMV is reported for the treatment of HCV chronic patients for a period of 3 months in single arm, open label study (11). A very

successful virologic response is obtained in HCV type 1 or 3 genotype patients by combination treatment of SOF and DCV for a period of 3 to 6 months. This combination has proven to be a successful and well tolerated treatment for patients as no or very few side or adverse effects were obtained. In the headline, the side effects were lethargy along with headache and may be nausea (12). In the present study we have optimized and validated a new, efficient, and economical RP-HPLC protocol for simultaneous determination and quantification of SOF and DCV both in solid dosage form as well as in human plasma.

METHODOLOGY

Chemicals

Sofosbuvir (99.9% pure) and daclatasvir dihydrochloride (purity 99.8%) were purchased from Jiangxi Synergy Pharmaceuticals, China. Potassium hexafluorophosphate, hydrochloric acid, phosphoric acid, sodium hydroxide and hydrogen peroxide were all from Sigma Aldrich (UK). HPLC grade acetonitrile, methanol and ethanol were supplied by Merck (USA).

Equipment

The HPLC analyses were carried out by Shimadzu gradient chromatography system LC20 (Japan) consisting of a quaternary pump LC-20AT, DGU-20A-5R degasser, CTO-20A column oven, SIL-20AHT auto-sampler and SPD-20A variable wavelength detector, controlled through Shimadzu Lab-Solutions software (version 6.43 SP1). Another HPLC system (Agilent 1260 Infinity, USA) was used for peak purity and repeated validation. It is comprised of quaternary pump 1260 Quat-Pump-VL, 1260 TCC column thermostat, 1260 ALS auto-sampler and diode array detector 1260 DAD-VL DAD controlled by Agilent Open-LAB software (version A.01.04). The column used was C18 (150×4.6 mm; 5µm) from Agela Technologies, USA.

Optimization of chromatographic conditions

Composition of mobile phase plays a very critical role in the analytical method development of any product. All the chromatographic conditions including flow rate, mobile phase composition, column oven temperature, and pH of buffer were optimized for the development of analytical method in

gradient mode. The composition of mobile phase was altered up-to 5% for the development of method and results were recorded against different compositions using acetonitrile and 0.02 M potassium hexafluorophosphate buffer with pH 2.5 to 3. The mobile phase composition with greater sensitivity, resolution, theoretical plates, and shorter run time was selected for method. The effect of column oven temperature was observed at 20 to 30°C and changes were recorded in the form of chromatograms. The results from the changes in flow rate of 1.0 to 1.5 ml min⁻¹ were recorded and the best flow rate was selected for development of analytical method.

The wavelength range used for detection of analytes was 275-285nm and 10 to 50 µL injection volume per sample was adjusted for the auto-sampler. The mobile phase was filtered through 0.22 µm nylon syringe filter and degassed in ultrasonic water bath for 30 min prior to use.

Preparation of standard solutions

The stock solution of standard was prepared in the concentration of 1.0 mg per mL for sofosbuvir and 0.15 mg per mL for daclatasvir in mobile phase and stored at 0-8 °C. To obtain the standard solutions for different analytical purposes, stock solution was diluted respectively by the same diluent.

Preparation of sample solution

Preparation of sample solutions was done by dissolving the sample in mobile phase and making the dilutions as required. To analyse the drugs in the blood, blood samples were taken from the hepatitis C patients. 6 ml of blood was taken from HCV patients taking sofosbuvir and daclatasvir tablets separately and transferred in the tubes containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant. These tubes were then centrifuged at 4000 rpm and 3ml plasma aliquot was taken from all the three tubes and make it three times of the original volume by acetonitrile. This mixture was shaken to precipitate the proteins and then centrifuged at rate of 4000 rpm to properly separate the precipitated proteins. The supernatant in quantity of 7 ml was taken and made 25 ml with acetonitrile. From this, two further dilutions were made taking 2 ml of this solution and

making 50 ml with mobile phase and taking 10 ml of this dilution and making 50 ml with mobile phase and all these three dilutions were subjected to analysis against a standard having concentration of sofosbuvir 10 µg and daclatasvir 1.5 µg.

Method validation

Method validation was performed by evaluating linearity, range, specificity, precision, accuracy, recovery, peak purity, sensitivity by limit of quantification (LOQ) and limit of detection (LOD), robustness, solution stability and system suitability.

Linearity

For both SOF and DCV, 10 standard solutions with different concentrations were prepared from the respective stock solution. These were injected in series and the peak areas were recorded. Calibration curves were constructed for individual concentrations against the corresponding peak areas.

Range

Range was also determined from the calibration curve using the maximum and minimum values of the straight line of the y-intercept.

Specificity

Samples of drug products were exposed to heat, light, acid, base, and oxidizing agent to produce degradation of the active ingredient for about 48 hours. The degraded samples were then analysed to observe any effect of interferences. The degradation conditions used included solutions of HCl (0.1, 1 and 2 N), NaOH (0.1, 1 and 2 N), H₂O₂ (1, 5 and 10%) and purified water.

Peak purity

Peak purity of six replicas of standard solutions were evaluated by injecting them according to the analytical method and assessing through DAD at different wavelengths at the same time.

Precision

Precision was determined based on repeatability and intermediate precision. For injection and analysis repeatability, six replicates of standard and sample solutions were analysed on three different days and the relative standard deviations (RSDs) were calculated. For intermediate precision, the same sample was analysed on two different equipment and the RSDs were recorded.

Accuracy

Accuracy was evaluated at three different levels by spiking the placebo prepared at 80, 100 and 120% and transferring respective amounts from the standard stock solution.

Sensitivity

The sensitivity of the method was analysed through LOD values and LOQ values. The LOD was determined by the residual standard deviation and slope of the line in range study and then experimentally verified. LOD and LOQ were measured by the formulas:

$$\text{LOD} = 3.3 \times \text{RSD}/m \text{ and } \text{LOQ} = 10 \times \text{RSD}/m$$

Where, m is the slope of line in linear relation and RSD is the residual standard deviation for the y intercept in the linear regression.

Robustness

The robustness for the method was evaluated to assess the effects of small but deliberate variations of the chromatographic conditions on the determination of SOF and DCV. Robustness was worked out by changing the flow rate (from 80-120%), temperature (to ±5°C) and the concentration of components in the mobile phase (to ±5%).

For solution stability, sample solutions were analysed within two hours of preparation and then analysed again after 24hrs following storage at 25°C and compared with freshly prepared standard solutions.

The system suitability was determined by calculating RSD, tailing factor, theoretical plates, and resolution.

RESULTS AND DISCUSSION

The results and discussion on this method are described below.

Optimization of chromatographic conditions

Optimization and validation of method was performed according to International Conference on Harmonization guidelines (ICH Q2R1).

Optimization of mobile phase

Although variability of mobile phase results symmetrical and well resolved peaks, however, a shift in the peaks retention time (RT) was observed. Initially various combinations of acetonitrile and water were tested and composition of 58:42 v/v (acetonitrile: water) was selected as optimum. In this composition the sofosbuvir showed noticeable retention but the daclatasvir showed no or very little retention.

The sofosbuvir was detected at 1.738 minutes and daclatasvir was detected at 0.990 minutes. To retain the daclatasvir, an ion pair reagent was used that should serve to retain the daclatasvir so that it may be identified purely. The ion pair reagent used was Potassium hexafluorophosphate. This reagent served to retain the daclatasvir from 1 minute to 8 minutes leading to maximum affinity of this molecule for the stationary phase. To make the sofosbuvir more retained in this composition of the mobile phase the pH of the mobile phase was reduced to 2.7 so that the sofosbuvir is retained from 2 minutes to 3 minutes helping in the purer and more precise identification and quantification of the drug.

Further increasing buffer concentration (from 50% to 60%) had a direct impact on increasing RT for both SOF and DCV (Fig.1). This effect was mainly demonstrated by DCV which showed a change of RT from 4.5 min to 9.3 min with a slight increase in the peak areas. The combination of acetonitrile: buffer at 42:58 v/v was found to be optimum (Fig. 1).

Optimization of buffer pH

With decrease in the buffer pH of the mobile phase from 3.0 to 2.5, the RT of the SOF increased from 2.0 to 2.9 min while there was only minor increase in RT of DCV (Fig.2.) with no observable effect on the peak areas in either case. The best resolution of peaks was observed at pH 2.7.

Optimization of flow rate

Increasing flow rate from 1.0 mL min⁻¹ to 1.5 mL min⁻¹ decreased RT (Fig.3). The optimal flow rate was 1.3 mL min⁻¹.

Optimization of column oven temperature

The alteration in ambient temperature from 20°C to 30°C had minimal effects on the RT, peak area, and resolution of peaks of both components (Fig.4.). For robust method development, ambient temperature was designated.

Method validation

Linearity

Table 1 shows the linearity of both for standard solution and spiked samples at ten levels of concentration ranging from 2-175 µg/mL for SOF and 0.3-26 µg/mL for DCV in the form of

correlation coefficient (r) and regression. The values from calibration curve suggest that method is quite linear in the described concentration range.

Percent Recovery (Accuracy)

Accuracy of the method was determined by calculating percent recovery. Percent recovery is calculated at three different spiked concentrations and was found to be 99% for both SOF and DCV.

Precision

Precision of newly developed method is determined by repeatability and intermediate precision. Also, the results of repeatability and intermediate precision expressed in RSD are presented in Table 1. Results for precision showed complete harmonization among analysis repeatability and injection repeatability.

Sensitivity (LOD and LOQ)

Values of LOD and LOQ for both drugs confirmed the method is very sensitive and can detect minimum quantity of drugs as low as 0.5 µg/mL and 0.075 µg/mL for SOF and DCV respectively.

System suitability

System suitability was analysed by RSD, symmetry, resolution, and number of theoretical plates.

Robustness

Minor changes in temperature, pH and flow rate did not significantly affect the peak area but slight shifts in retention time were observed. Robustness for the SOF and DCV are revealed in Table 1.

Specificity

Peak purity was taken on automatic spectral wavelengths by Diode Array Detector and showed that both SOF and DCV peaks were pure. Peak purity of SOF was calculated by 43 spectra and all the spectra fell in the calculated threshold limit (999.944) and the purity factor was 999.971. DCV peak purity was calculated at 85 spectra and all the spectra fell in threshold limit which was 999.949 and the purity factor was 999.975. The graphical representation for peak purity by diode array detector for Sofosbuvir and Daclatasvir is shown in figure 5 and 6 respectively.

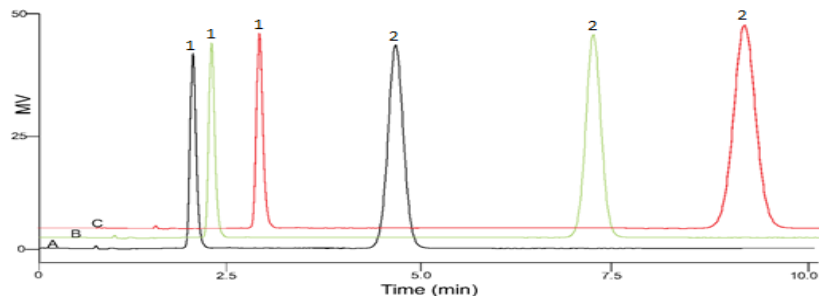


Figure 1. Peaks of SOF (1) and DCV (2) obtained at mobile phase buffer concentrations of 55% (A), 58% (B) and 60% (C).

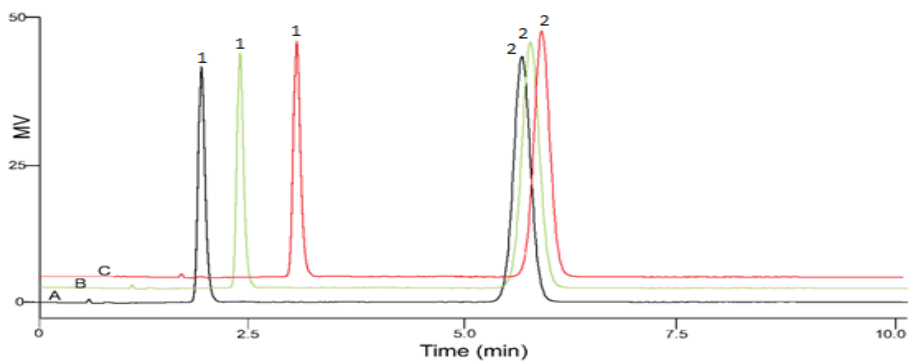


Figure 2. RT of SOF (1) and DCV (2) at pH 3.0 (A), pH 2.7 (B) and at pH 2.5 (C) of the mobile phase.

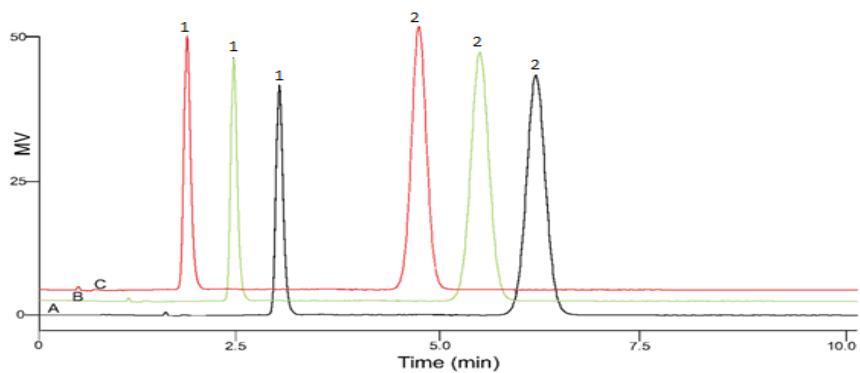


Figure 3. Retention time, area, and peak height of SOF (1) and DCV (2) (A) at flow rate 1.0 mL min⁻¹ (B) at flow rate 1.3 mL min⁻¹ (C) at flow rate 1.5 mL min⁻¹

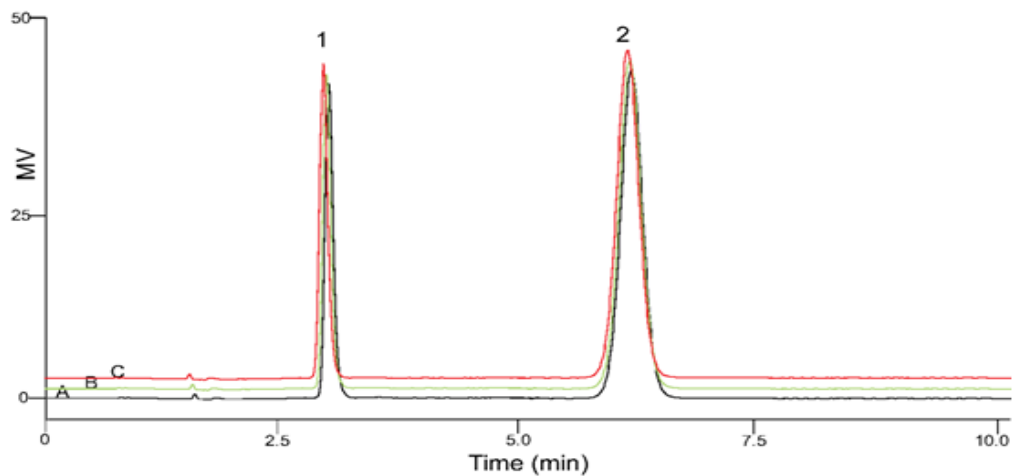


Figure 4. Retention time, peak area, and resolution of SOF (1) and DCV (2) (A) at 20°C. (B) 25°C. (C) at 30°C.

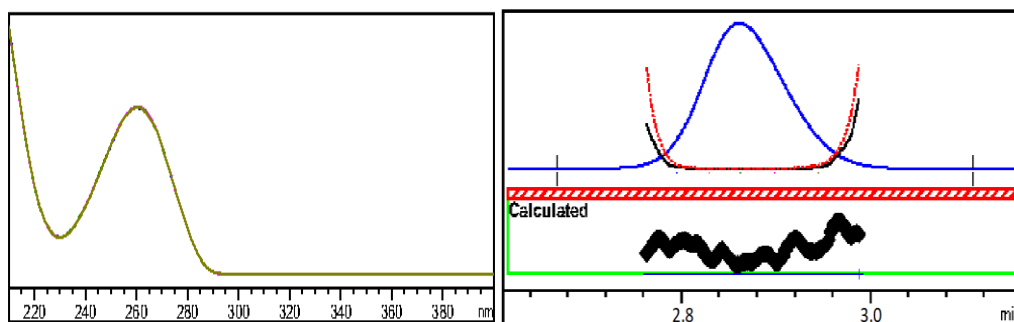


Figure 5. Peak purity of sofosbuvir

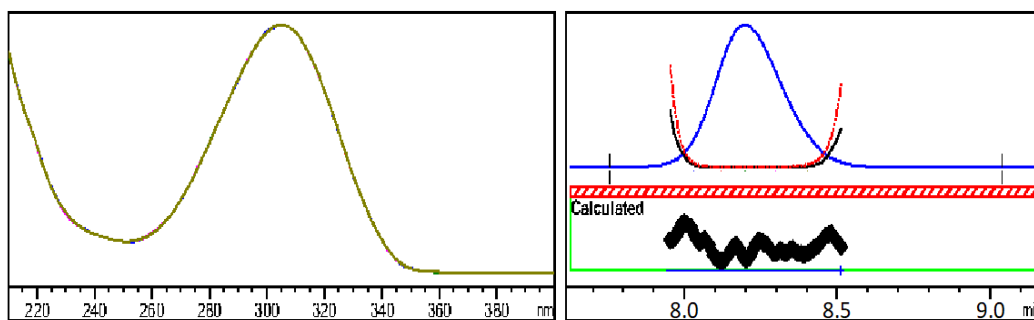


Figure 6. Peak purity of Daclatasvir

Table 1. Range, linearity, accuracy, precision, sensitivity, robustness, system suitability, solution stability

Sr. #	Parameters	SOF	DCV
1	Concentration range ($\mu\text{g/mL}$)	2.0-175	0.3-26.25
2	Linearity		
	Regression equation	$y = 2701.1x + 318.47$	$y = 39847x - 1286.6$
	Correlation coefficient	$r^2 = 0.9997$	$r^2 = 0.9998$
3	Accuracy (Average Recovery %, RSD)		
	Spiked conc. (80%)	97.72, 0.17	97.39, 0.16
	Spiked conc. (100%)	98.29, 0.30	98.18, 0.46
	Spiked conc. (120%)	99.15, 0.29	99.35, 0.23
4	Precision (average peak area, RSD)		
	Injection repeatability	399167, 0.31	799294, 0.28
	Analysis repeatability	402817, 0.29	806280.33, 0.29
	Intermediate precision	406235, 0.42	813861.67, 0.32
5	Sensitivity ($\mu\text{g/mL}$)		
	LOD	0.5	0.075
	LOQ	2	0.3
6	Robustness (RSD% of peak areas)		
	Percentage of buffer (55,58,60)	0.32	0.26
	pH of Buffer (2.5, 2.7, 3.0)	0.51	0.43
	Flow rate, mL min^{-1} (1.0, 1.3, 1.5)	0.27	0.19
	Column oven temperature in $^{\circ}\text{C}$ (20, 25, 30)	0.16	0.23
7	System Suitability		
	Theoretical plates	2035	3429
	Symmetry	1.66	1.37
	Resolution		9.28
	RSD (%)	0.614	0.463
8	Solution stability (average recovery %, RSD)		
	Fresh Standard	100, 0.61	100, 0.46
	Spiked Sample	100.29, 0.29	100.04, 0.29
	24 hrs. Standard	100, 0.39	100, 0.44
	Spiked Sample	99.15, 0.30	99.44, 0.46

Application of the method

SOF and DCV combination therapy is significant for the treatment of hepatitis C. Development of this RP-HPLC method for the simultaneous determination and quantification of SOF and DCV is a part of an extensive project of novel formulation development (fixed dose combination tablets of SOF and DCV). This method is successfully applied for raw materials as well as in combination tablets. This method may also be applied for the quantification of sofosbuvir and daclatasvir in blood samples of patients taking these two antivirals for hepatitis C treatment.

CONCLUSION

The present study investigated the first RP-HPLC method for the simultaneous determination and quantification of SOF and DCV. This method is easy, economical, sensitive, precise, and accurate. The proposed method has not only been found appropriate for routine analysis in the manufacturing unit for the determination of raw materials as well as dosage form, but it may also be suitable for analysis of sofosbuvir and daclatasvir in human plasma for research purposes.

ACKNOWLEDGEMENT

None

DECLARATIONS

Authors' Contributions

SHM contributed to the study concept; AUS, KS, and NHK contributed to the study design and data collection. SHM, TEM, and SA contributed to the data analysis and interpretation. FK and MM did the literature review and critically reviewed the manuscript. All the authors read and approved the final manuscript.

Ethical Approval

Not applicable

Conflict of Interest

The authors declared no conflict of interest among them.

REFERENCES

1. Elkhodairy KA, Hassan MA, Afifi SA. Formulation and optimization of orodispersible tablets of flutamide. Saudi Pharmaceutical Journal. 2014;22(1):53-61.
<https://doi.org/10.1016/j.jsps.2013.01.009>

2. Shah N, Pierce T, Kowdley KV. Review of direct-acting antiviral agents for the treatment of chronic hepatitis C. Expert opinion on investigational drugs. 2013;22(9):1107-21.
<https://doi.org/10.1517/13543784.2013.806482>

3. Miura M, Maekawa S, Sato M, Komatsu N, Tatsumi A, Takano S, et al. Deep sequencing analysis of variants resistant to the non-structural 5 A inhibitor daclatasvir in patients with genotype 1b hepatitis C virus infection. Hepatology Research. 2014;44(14):E360-E7.
<https://doi.org/10.1111/hepr.12316>

4. Obaid H, Sohaib AU, Munir AB, Akbar J, Zaman MQ-u, Amjad MA, et al. Prescription Errors Still a Challenge in Pakistan-A Cross-Sectional Study from Central Punjab, Pakistan. RADS Journal of Pharmacy and Pharmaceutical Sciences. 2019;7(4):181-5.
<https://doi.org/10.37962/jpps.v7i4.327>

5. Bhatia HK, Singh H, Grewal N, Natt NK. Sofosbuvir: A novel treatment option for chronic hepatitis C infection. Journal of pharmacology and pharmacotherapeutics. 2014;5(4):278-84.
<https://doi.org/10.4103/0976-500X.142464>

6. Nelson DR, Cooper JN, Lalezari JP, Lawitz E, Pockros PJ, Gitlin N, et al. All-oral 12-week treatment with daclatasvir plus sofosbuvir in patients with hepatitis C virus genotype 3 infection: ALLY-3 phase III study. Hepatology. 2015;61(4):1127-35.
<https://doi.org/10.1002/hep.27726>

7. Csermely P, Korcsmáros T, Kiss HJ, London G, Nussinov R. Structure and dynamics of molecular networks: a novel paradigm of drug discovery: a comprehensive review. Pharmacology & Therapeutics. 2013;138(3):333-408.
<https://doi.org/10.1016/j.pharmthera.2013.01.016>

8. Gane EJ, Stedman CA, Hyland RH, Ding X, Svarovskaia E, Subramanian GM, et al. Efficacy of nucleotide polymerase inhibitor sofosbuvir plus the NS5A inhibitor ledipasvir or the NS5B non-nucleoside inhibitor GS-9669 against HCV genotype 1 infection. Gastroenterology. 2014;146(3):736-43. e1.
<https://doi.org/10.1053/j.gastro.2013.11.007>

9. Asghar R, Zeeshan N, Sohaib AU, Bakar Munir A, Amjad MA, Ahmad S, et al. Assessment of Knowledge and Practices of

Voluntary Blood Donation Among Pharmacy Students; A Cross-Sectional Survey from Lahore, Pakistan. RADS Journal of Pharmacy and Pharmaceutical Sciences. 2019;7(4):206-14.

<https://doi.org/10.37962/jpps.v7i4.337>

10. Guedj J, Pang PS, Denning J, Rodriguez-Torres M, Lawitz E, Symonds W, et al. Analysis of hepatitis C viral kinetics during administration of two nucleotide analogues: sofosbuvir (GS-7977) and GS-0938. Antiviral therapy. 2014;19(2):211-20.

<https://doi.org/10.3851/IMP2733>

11. Pellicelli AM, Montalbano M, Lionetti R, Durand C, Ferenci P, D'Offizi G, et al.

Sofosbuvir plus daclatasvir for post-transplant recurrent hepatitis C: potent antiviral activity but no clinical benefit if treatment is given late. Digestive and Liver Disease. 2014;46(10):923-7.

<https://doi.org/10.1016/j.dld.2014.06.004>

12. Rocca LG, Yawn BP, Wollan P, Kim WR. Management of patients with hepatitis C in a community population: diagnosis, discussions, and decisions to treat. The Annals of Family Medicine. 2004;2(2):116-24.

<https://doi.org/10.1370/afm.62>



Online Research Publications by Authors is licensed under a Creative Commons Attribution-Non Commercial-No Derivatives 4.0 International License.