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Multivariate optimization and evaluation of quaternary mixture in bulk and co-formulated dosage forms by central composite design

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Abstract

Background: The current study describes the use of central composite design for multivariate optimization of resolution and retention time, taking into account different critical method parameters like organic phase, pH, flow rate, and wavelength for risk assessment. The chromatographic method for the assay of the most effective anti-viral regimen (EPCLUSA, DARVONI, and HARVONI) was developed. An experimental design was presented by sequential investigation of four independent parameters. The method was developed using XTERRA C18 (250 mm × 4.6 mm, 5 μm particle size) column in isocratic mode using potassium dihydrogen phosphate buffer (pH adjusted to 5) and acetonitrile (50:50 % v/v) as mobile phase at a flow rate of 1.0 ml/min and UV detection wavelength of 260 nm.

Results: The separation of four drugs with fine resolution and preferable retention times was achieved. Retention times of four drugs were found to be 2.96, 3.91, 7.15, and 11.94 min for daclatasvir, sofosbuvir, velpatasvir, and ledipasvir, respectively. The percentage accuracy of labelled claim was in the range of 99–102%, and the pooled %RSD for repeatability, precision, and accuracy was less than 2%.

Conclusion: The suggested method was applied for quantification and identification of studied drugs in tablets; the results agreed with the label claim and were validated according to the ICH guidelines. The optimized method can be used for pharmacokinetic and quality control studies.

Keywords: AQbD, HPLC, Sofosbuvir, Velpatasvir, Ledipasvir, Daclatasvir, EPCLUSA, DARVONI, HARVONI, Anti-viral drugs

Background

The fast-developing resistance to anti-viral drugs due to rapid viral cell mutation in HIV and hepatitis C virus has garnered researchers worldwide to focus on combination therapy and in analysing the efficacy and toxicity of drugs that act on different targets. Over the past two decades, the researchers worked immensely to identify new molecular targets (structural and non-structural proteins), and trials were still going on to develop drugs which were selectively toxic to viruses without causing harm to the host cell. Researchers also aided in improving the efficacy of study by developing HCV replicon cell culture lines for pharmacokinetic and clinical investigations. Despite all the advancements, the anti-viral drug research was still running at a slower pace than expected, so it is the due responsibility of the analytical chemist to develop a method for simultaneous estimation of anti-viral drugs in clinical samples and dosage forms, to make the analysis of drug cost-effective, time-saving, and efficient to demarcate the toxic and therapeutic doses of drugs. The complexity involved in the analysis of effective anti-viral treatment regimen was

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eased by developing a single simple, robust, and selective analytical method for simultaneous assessment of anti-viral drugs such as sofosbuvir, velpatasvir, ledipasvir, and daclatasvir using quality-based drug design principles.

HCV therapy was initially started using a combination of pegylated interferon and ribavarin drugs, until protease inhibitors boceprevir and telaprevir were identified as potent targeted therapies. Later, the advancement in diagnostic tools and molecular target discovery (NS5A, NS5B, and NS3/4A) brought to light drugs like simprevir. But the intense results in therapy were noticed only after the discovery of sofosbuvir. This potent molecule was now available in combination with different drugs such as velpatasvir, daclatasvir, and ledipasvir to treat hepatitis [1–3].

Sofosbuvir is a crystalline solid substance, white to off-white in colour, and named as "(S)-Isopropyl, 2-((S)-(((2R, 3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyri-midin-1(2H)-yl)-4-fluoro-3-hydroxy-4-methyl tetra hydrofuran-2-yl)methoxy)-(phenoxy) phosphorylamino) propanoate". It inhibits the synthesis of RNA by targeting the NS5B polymerase protein of hepatitis C virus. Sofosbuvir is co-formulated in many multi-drug anti-viral formulations due to its high tolerance to resistance [4].

Ledipasvir named as "(2S)-1-[(6S)-6-[5-(9,9-difluoro-7-{2-[(1R,3S,4S)-2-[(2S)-2{[hydroxyl(methoxy)methylidene]amino}-3-ethylbutanoyl]-2-azabicyclo[2.2.1] heptan-3-yl]-1H-1,3-benzodiazol-6-yl}-9H-fluoren-2-yl)-1H-imidazol-2-yl]-5-azaspiro[2.4]heptan-5-yl]-2-{[hydroxy(-methoxy) methylidene]amino}-3-methylbutan-1-one" is an inhibitor of the NS5A protein of hepatitis C virus (HCV) which plays a major role in the assembly of HCV virions and viral RNA replication [5].

Velpatasvir is a white solid substance and chemically known as "(2S)-2-{[hydroxyl (methoxy) methylidene] amino}-1-[(2S,5S)-2-(17-{2-[(2S,4S)-1-[(2R)-2-{[hydroxyl (methoxy) methylidene] amino}-2-phenylacetyl]-4-(methoxymethyl)pyrrolidin-2-yl]-1H-imidazol-5-yl}-21-oxa-5,7-diazapentacyclohenicosa-1(13),2,4(8),6,9,11,14(19),15,17-nonaen-6-yl)-5-methyl pyrrolidin-1-yl]-3-methylbutan-1-one".The drug inhibits viral replication by competing with RNA to bind at domain I of NS5A consisting of amino acids [6, 7].

Daclatasvir is beige to white powdery substance and chemically known as "methyl *N*-[(2S)-1-[(2S)-2-[5-(4′-{2-[(2S)-1-[(2S)-2-[(methoxycarbonyl) amino]-3-methylbutanoyl]pyrrolidin-2-yl]-1H-imidazol-5-yl}-[1,1′-biphenyl]-4-yl)-1H-imidazol-2-yl]pyrrolidin-1-yl]-3-methyl-1-oxobutan-2-yl]carbamate" and functions by disrupting the hyper-phosphorylated NS5A proteins, thus interferes with new HCV replication complexes function [8].

Quality by design (QbD) helps in understanding and monitoring the process based on quality risk management and sound science [9]. This method also offers a good regulatory flexibility compared to normal one factor at a time (OFAT) experiments [10]. QbD provides information about significant parameters of the method and their interactions in a broader prospect [11, 12]. It helps to create a method operable design region which eases the work of the analytical chemist and regulatory bodies [13–15]. It also reduces the cost, analysis time, and the number of experimental trials [16, 17]. The quality of HPLC methods has become increasingly important in a QbD environment [18, 19]. Thorough literature studies indicated the availability of methods for the analysis of drugs individually or as a combination in a binary mixture, plasma or dosage form, but to date, no analytical method was tried to assay this quaternary mixture in bulk or dosage forms [20–26]. Thus, this article portrays important concepts, such as central composite design to analyse the four anti-viral drugs, i.e. sofosbuvir, velpatasvir, ledipasvir, and daclatasvir by RP-HPLC.

Methods

Chemicals and apparatus

The Shimadzu HPLC (Detector model: SPD20A, Pump model: LC-20 AD) with a UV-visible detector was used for the method development and quantification. LC solutions software was used for processing and monitoring the output signal. The chromatographic column XTERRA RP-18 (250 mm × 4.6 mm, 5 μm), rheodyne syringe loading manual sample injector (20 μl) was used for the analysis of drugs. The design used for the analysis of LC experiments was the Sigma Tech analytical QbD software. Shimadzu AUX 220 model analytical balance, Elico pH meter, and REMI model Centrifuge were utilized for this work. API (sofosbuvir, velpatasvir, ledipasvir, and daclatasvir) were obtained as a gift sample from Hetero Drugs Private Limited. Analytical grade potassium dihydrogen orthophosphate, sodium hydroxide, potassium hydroxide, di-sodium hydrogen orthophosphate, HPLC grade acetonitrile, and methanol were acquired from Merck, Mumbai. Pharmaceutical tablet dosage forms such as ECLUPSA, HARVONI, and DARVONI were procured from local pharmacies.

Preparation of mobile phase

Acetonitrile and potassium dihydrogen phosphate buffer, pH adjusted to 5.0, was taken in the ratio of 50:50 % v/v and filtered through a 0.45-μm membrane filter and sonicated to degas. Acetonitrile and water in the ratio of 50:50 % v/v were used as diluents.

Preparation of standard solution

Ten milligrams of each standard drug was accurately weighed and transferred into four different 10-ml volumetric flasks, followed by the addition of 7 ml diluent to dissolve. The solution was then sonicated for 15 min and

diluted to the mark with diluent to prepare a 1000-μg/ml concentrated solution.

Chromatographic conditions

In order to study the effect of controlled input factors on responses, they were systematically varied in an experimental design known as "design of experiment" (DOE). DOE was twofold and comprises screening and optimization carried out sequentially [27]. Screening focuses on discriminating the critical method parameters using screening designs such as two-level full or fractional factorial designs in most cases. These designs screen the method parameters that have the ability to affect the analytical method response. The full and fractional design will explore many parameters by setting each on two levels, i.e. higher and lower. Its conclusion helps in identifying the most significant parameters among many others that were involved in the design.

Four variables were selected with their lower and upper values, using the Sigmatech software for screening, and 16 experimental trials were obtained. Based on the results, some of the variables were selected and fixed for the optimization. The selected significant factors are further studied using more comprehensive designs to set the most influential factors at levels that enhance analytical CQAs simultaneously. It provides a base for scientific understanding of the relation between quantities of input variables (CMP) and output response which will show a considerable effect on the method performance and ATP [17, 19]. The central composite design was utilized to determine the significant variables and optimize the chromatographic conditions with the lowest number of runs. From the screening results, wavelength and organic phases were fixed as dependent variables, and % of organic phase, aqueous phase pH, and flow rate were selected as variables for optimization with its lower and upper values reported in Table 1. These variables were placed in the Sigmatech software, and 8 experimental trials were obtained. Based on the results, a method was developed with the optimized conditions. The chromatographic trials suggested by the system were performed using Shimadzu HPLC with XTERRA RP 18 (250 mm × 4.6 mm × 5 μ) column at UV detection wavelength 260 nm.

Table 1 Variables for optimization with its upper and lower values (qualitative)

S.No	Variable	Variable name	Units	Low value	High value
1	X_1	Organic phase	%	30	70
2	X_2	Buffer (pH)	Number	4	6
3	X_3	Flow rate	ml/min	0.6	1

Method validation

The method operable design region (MODR) constructed from contour plots controls the variations in the response [11, 28]. The optimized chromatographic method parameters were verified and validated as per standard guidelines [29–32].

Specificity

The spectra obtained from the synthetic mixture of standard solutions and commercial formulations were compared to assess the specificity of the method [33]. The chromatograms of blank, placebo, standard, and commercial formulations were examined to determine any additional peaks appearing at the retention time of sofosbuvir, velpatasvir, ledipasvir, and daclatasvir.

Linearity and range

Sofosbuvir aliquots of 0.6, 1.2, 1.8, 2.4, 3.0, and 3.6 ml were withdrawn from the stock solution 1000 μg/ml and were diluted to 10 ml such that the final concentration of sofosbuvir is in the range of 60–360 μg/ml. Velpatasvir and ledipasvir aliquots of 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 ml were withdrawn from their respective stock solution 1000 μg/ml and diluted to 10 ml with diluent such that the final concentration of velpatasvir and ledipasvir was in the range of 40–140 μg/ml. Daclatasvir aliquots of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 ml were withdrawn from the stock solution 1000 μg/ml and diluted to 10 ml with diluent such that the final concentration of daclatasvir in the range of 20–120 μg/ml was obtained; 20 μl of each concentration was injected, and a calibration curve was plotted by taking peak area on the Y-axis and concentration of drug on the X-axis.

Accuracy

The accuracy of the method was determined by spiking known concentration of standard drugs (80%, 100%, and 120%) to their respective formulation three times in a sequence. The parameters like percent recovery and per cent relative standard deviation of all the four drugs were calculated accurately to justify the study.

Precision

The drugs sofosbuvir (60,180, and 360 μg/ml), velpatasvir (40, 80, and 140 μg/ml), ledipasvir (40, 80, and 180 μg/ml), and daclatasvir (20, 60, and 120 μg/ml) were injected at each concentration level, and their peak areas were noted in triplicate on the same day and on three different days of the same week for intra- and inter-day precision studies. Parameters like per cent relative standard deviation in peak area and retention time of corresponding drug peaks were calculated as part of the validation.

Table 2 System response of sofosbuvir, velpatasvir, ledipasvir, and daclatasvir in complete composite design

S.No	Combination	% organic phase	pH	Flow rate	Theoretical (sofosbuvir)	Theoretical (velpatasvir)	Theoretical (ledipasvir)	Theoretical (daclatasvir)
		X_1	X_2	X_3				
1	1	30	4.0	0.6	17,398.754	921	955	934
2	X_1	70	4.0	0.6	2845.40	7132.22	4852.92	1506.5
3	X_2	30	6.0	0.6	19,215.652	932	965	956
4	X_1X_2	70	6.0	0.6	8357.634	8403.91	5528.55	7649.63
5	X_3	30	4.0	1.0	12,942.93	984	964.5	990
6	X_1X_3	70	4.0	1.0	2841.49	5047.30	3683.29	4144.34
7	X_2X_3	30	6.0	1.0	15,081.284	989	978	949
8	$X_1X_2X_3$	70	6.0	1.0	5090.77	5660.94	3738.79	4541.39

LOD and LOQ

From the linearity data, the limit of detection and quantification was calculated using the following formula.

$$LOD = \frac{3.3\sigma}{S}$$

$$LOQ = \frac{10\sigma}{S}$$

where σ is the standard deviation of the response, and S is the slope of the calibration curve of the analytes.

Robustness

To estimate the robustness of the experimental design, optimized chromatographic parameters like flow rate (± 0.1 ml/min), pH (±0.2), % organic phase (±2%), and wavelength (±2 nm) were varied slightly in the method used for the analysis of samples containing sofosbuvir, velpatasvir, ledipasvir, and daclatasvir.

Assay of marketed formulation

The marketed formulations 20 tablets each of the respective drugs, EPCLUSA (sofosbuvir 400 mg + velpatasivir 100 mg), HARVONI (sofosbuvir 400 mg + ledipasivir 100 mg), and DARVONI (sofosbuvir 400 mg + daclatasivir 60 mg) were weighed, and average weight of each tablet was noted. An estimated quantity of powder equivalent to 50 mg velpatasvir and 200 mg sofosbuvir in EPCLUSA (520.88 mg), 50 mg ledipasvir and 222 mg sofosbuvir in HARVONI (579.21 mg), and 30 mg of daclatasvir and 200 mg of sofosbuvir in DARVONI (384.19 mg) was taken in 25 ml volumetric flasks; 20 ml of acetonitrile was added initially to all the flasks and sonicated for 15 min; later, the flasks were brimmed up to the mark using acetonitrile to prepare stock solutions. The stock solutions were then filtered using Whatman filter paper (No: 41), and sample solutions were prepared by diluting 1:10 with diluents to obtain 200 μg/ml of velpatasvir and 800 μg/ml of sofosbuvir for Epclusa, 200 μg/ml of ledipasvir and 888 μg/ml of sofosbuvir for HARVONI, and 120 μg/ml of daclatasvir and 800 μg/ml of sofosbuvir for DARVONI. Further dilution of sample solutions within a range of linearity was undertaken by taking 3 ml of solution from respective volumetric flasks and making up to 10 ml with diluents to obtain 60 μg/ml of velpatasvir and 240 μg/ml of sofosbuvir for EPCLUSA, 60 μg/ml of ledipasvir and 252 μg/ml of sofosbuvir for HARVONI, and 36 μg/ml of daclatasvir and 240 μg/ml of sofosbuvir for DARVONI

Table 3 Statistical optimization analysis of sofosbuvir, velpatasvir, ledipasvir, and daclatasvir

S.No	Combination	Sofosbuvir		Velpatasvir		Ledipasvir		Daclatasvir	
		Coefficient	SS ratio	Coefficient	SS ratio	Coefficient	SS ratio	Coefficient	SS ratio
1	b_0	10,471.73	–	3758.796	–	2708.256	–	2708.857	–
2	b_1	−5687.915	85.8153%	2802.296	90.1972%	1742.631	90.942%	1751.607	56.360%
3	b_2	1464.595	5.6898%	237.662	0.6488%	94.328	0.2665%	815.147	12.206%
4	b_{12}	475.782	0.6004%	233.662	0.6271%	88.453	0.2343%	819.897	12.348%
5	b_3	−1482.62	5.8307%	−588.4863	3.9778%	−367.111	4.036%	−52.675	0.051%
6	b_{13}	664.927	1.1728%	−618.486	4.3936%	−372.736	4.1606%	−64.925	0.0774%
7	b_{23}	−367.687	0.3586%	−83.006	0.0791%	−77.078	0.1779%	−726.135	9.685%
8	b_{123}	−448.05	0.5325	−81.506	0.0763%	−77.953	0.182%	−710.385	9.2702%

SS sum of squares

Table 4 Central composite plan of anti-viral drugs

S.No.	Trials	Organic phase	pH	Flow rate	Responses (Sof + Vel + Led + Dac)			
		X_1	X_2	X_3	TP_1	TP_2	TP_3	TP_4
1	I	30	4.0	0.6	17,398.754	921	955	934
2	X_1	70	4.0	0.6	2845.40	7132.22	4852.92	1506.5
3	X_2	30	6.0	0.6	19,215.652	932	965	956
4	X_1X_2	70	6.0	0.6	8357.634	8403.91	5528.55	7649.63
5	X_3	30	4.0	1.0	12,942.93	984	964.5	990
6	X_1X_3	70	4.0	1.0	2841.49	5047.30	3683.29	4144.34
7	X_2X_3	30	6.0	1.0	15,081.284	989	978	949
8	$X_1X_2X_3$	70	6.0	1.0	5090.77	5660.94	3738.79	4541.39
9	Mid-point	50	5.0	0.8	8167.088	7808.8	1678.658	6681.036
13	$X_1 - 2L$	10	5.0	0.8	591.83	491.08	521.66	738.19
14	$X_1 + 2L$	90	5.0	0.8	5766.10	444.42	632.16	569.14
15	$X_2 - 2L$	50	3.0	0.8	6269.09	4009.30	1688.17	1864.41
16	$X_2 + 2L$	50	7.0	0.8	7678.7	3967.22	541.32	4069.66
17	$X_3 - 2L$	50	5.0	0.4	10,603.02	8545.68	3623.83	6023.1
18	$X_3 + 2L$	50	5.0	1.2	5649.47	4589.46	1650.81	3099.35

TP Theoretical plates

Results

Preliminary studies

Authentication of drugs was done by determination of melting point, UV absorption spectra (λ_{max}), and FT-IR. The results of these studies were compared with the reference values and found to be within the limit of acceptance. The individual standard solutions (10 µg/ml) of four drugs in diluent (acetonitrile: water, 50:50 % v/v) were analysed in a UV spectrophotometer ranging from 200 to 400 nm. From the overlain spectrum of four drugs, the wavelength 260 nm was selected for estimation in order to get maximum responses for four analytes [29, 33].

Screening studies

The critical process parameters such as organic phase, aqueous phase (pH), flow rate, wavelength, and interactions between the factors, which can affect the chromatographic responses such as theoretical plate number and retention time, were screened by using experimental design.

AQbD approach method optimization

In order to determine optimum chromatographic conditions, selected variables were screened using a factorial experimental design (2^3). Software-designed experimental

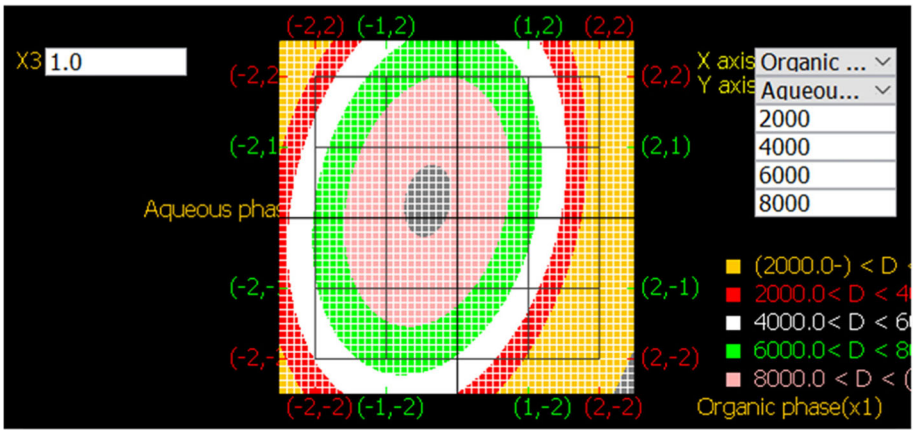

Fig. 1 Contour plot of sofosbuvir at 1.0 ml flow rate

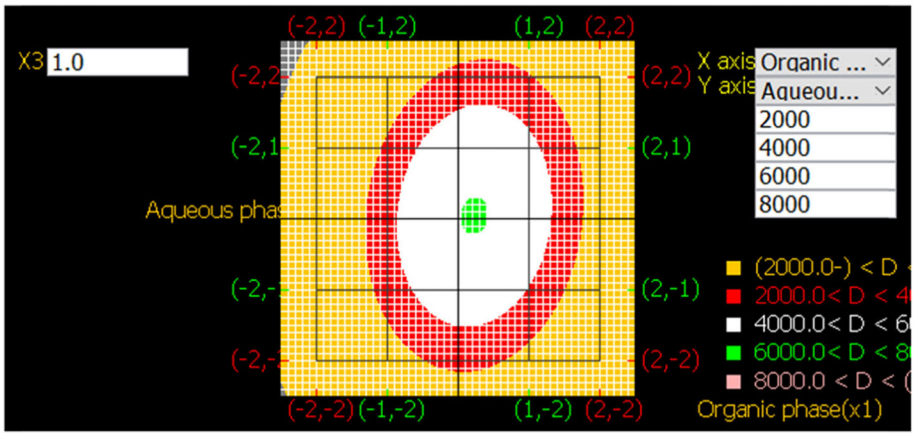

Fig. 2 Contour plot of velpatasvir at 1.0 ml flow rate

trials were performed with respect to three variables such as % organic phase (X_1), aqueous phase pH (X_2),and flow rate (X_3) to obtain theoretical plate number as method control response for four drugs. The software suggested central composite experimental design (CCD) was evaluated for the effect of individual factors on the response in the form of a contour plot.

Statistical analysis

Statistical analysis of experimental observations was performed to evaluate significant factors that affect the chromatographic response and were tabulated in Tables 2 and 3. Polynomial equations for the prediction of responses were obtained as follows (Eqs. 1 to 4) for sofosbuvir, velpatasvir, ledipasvir, and daclatasvir.

$$Y_{SOF} = 10471.73 + (-5687)b_1 + (1464)b_2 + (475.782)b_{12} + (-1482.62)b_3 + (-664.927)b_{13} + (-367.687)b_{23} + (-448.05)b_{123} \quad (1)$$

$$Y_{VEL} = 3758.796 + (2802.296)b_1 + (237.662)b_2 + (233.662)b_{12} + (-588.486)b_3 + (-618.482)b_{13} + (-83.006)b_{23} + (-81.506)b_{123} \quad (2)$$

$$Y_{LED} = 2708.256 + (1742.631)b_1 + (94.328)b_2 + (88.453)b_{12} + (-367.111)b_3 + (-372.736)b_{13} + (-77.078)b_{23} + (-77.953)b_{123} \quad (3)$$

$$Y_{DAC} = 2708.857 + (1751.607)b_1 + (815.147)b_2 + (819.897)b_{12} + (-52.675)b_3 + (-64.925)b_{13} + (-726.135)b_{23} + (-710.385)b_{123} \quad (4)$$

(Y—response of respective drug; b_0—intercept; b_1, b_2, and b_3—regression coefficients of variables X_1, X_2, and X_3, respectively; b_{12}, b_{13}, and b_{23}—regression coefficients for two factor interactions between variables; and b_{123}—

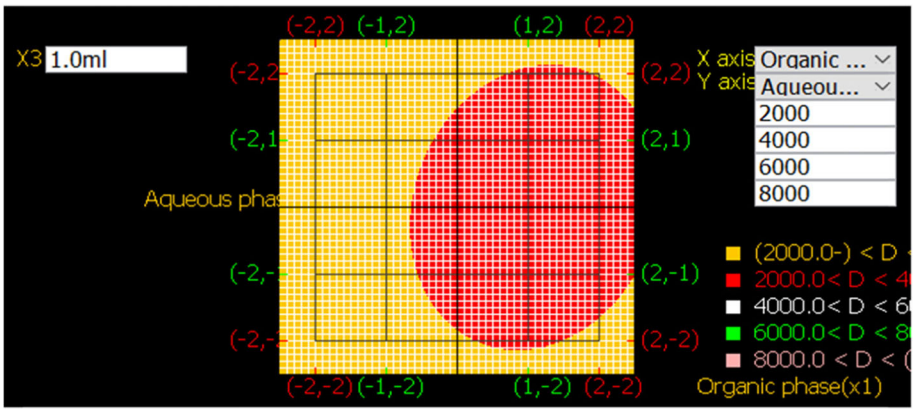

Fig. 3 Contour plot of ledipasvir at 1.0 ml flow rate

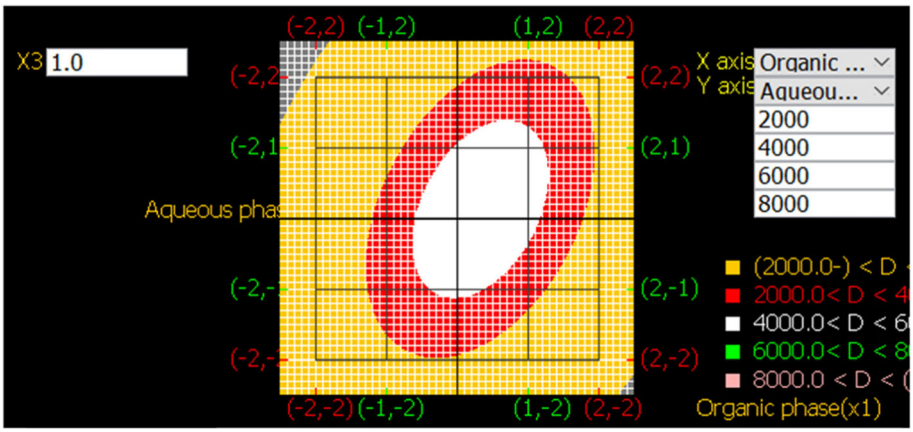

Fig. 4 Contour plot of daclatasvir at 1.0 ml flow rate

coefficient for three factor interactions between three variables).

Statistical data analysis of sofosbuvir exhibited X_1 (% organic phase) as the significant factor owing to the highest SS ratio (85.815%) at b_1 combination. The negative sign of the coefficient indicates that the lower the % organic phase, the higher is the plate number of the chromatographic system. The next best SS ratio (5.8307%) was obtained at b_3 combination, i.e. X_3 variable, so the flow rate was also an important factor. The negative sign of the coefficient indicates that the lower the flow rate, the higher the response of the chromatographic system. The SS ratio (5.689%) was obtained at b_2 combination, i.e. X_2 variable, so the pH was also an important factor.

Statistical data analysis of velpatasvir, ledipasvir, and daclatasvir exhibited X_1 (% organic phase) as the significant factor owing to the highest SS ratios (90.19%, 90.94%, and 56.36%) at b_1 combination. The positive sign of the coefficient indicates that higher the % organic phase in the mobile phase, the higher is the response (theoretical plates number) of the chromatographic system. The SS ratio of velpatasvir and ledipasvir (4.39% and 4.16%) was obtained at b_{13} combination, i.e.; X_1X_3 variable, so the interaction of % organic phase (X_1) and flow rate (X_3) has an influence on the chromatographic system. The negative sign of the coefficient indicates that the lower the interaction, the better the response of the chromatographic system. The SS ratio of daclatasvir (12.206%) indicated pH as an influential factor.

Analysis of results (ANOVA) showed curvature effect was significant. The curvature effect showed a 95% confidence level. This indicates X_1, X_2, and X_3 along with interactions were highly significant at a 95% confidence level. Hence, it is mandatory to select a central composite design. The central composite design of sofosbuvir, velpatasvir, ledipasvir, and daclatasvir with its responses (theoretical plate number) was reported in Table 4.

Table 5 Optimized chromatographic conditions

Parameter/conditions	Description/values
Column	XTERRA RP18 (250 × 4.6 mm, 5 μ)
Detector	UV-Vis detector
Flow rate	**1.0 mL/min**
Injection volume	20 μl
Wavelength	260 nm
Column temperature	25 °C
Run time	15 min
Buffer	Phosphate buffer (pH adjusted to 5.0)
Mobile phase	**ACN: buffer (50:50)**
Program	Isocratic

Contour plot

The method was optimized based on contour plots drawn by varying % organic phase and pH keeping flow rate constant. The plots are as shown in Figs. 1, 2, 3, and 4. The % organic phase has a prominent effect on theoretical plate number for the four drugs and indicates that organic modifier above + 1 level (70%) and below −1 level (30%) decreases the response. The aqueous phase (pH) of the mobile phase showed a significant effect on the four drugs and indicates that pH above + 1 (7) and below −1 (4) decreases the response. Hence, % organic phase at mid-level and the pH at a lower level can be preferred to reduce the cost of the experiment.

Optimized experimental conditions

To optimize the HPLC parameters, several mobile phase compositions, changes in pH, and changes in flow rate

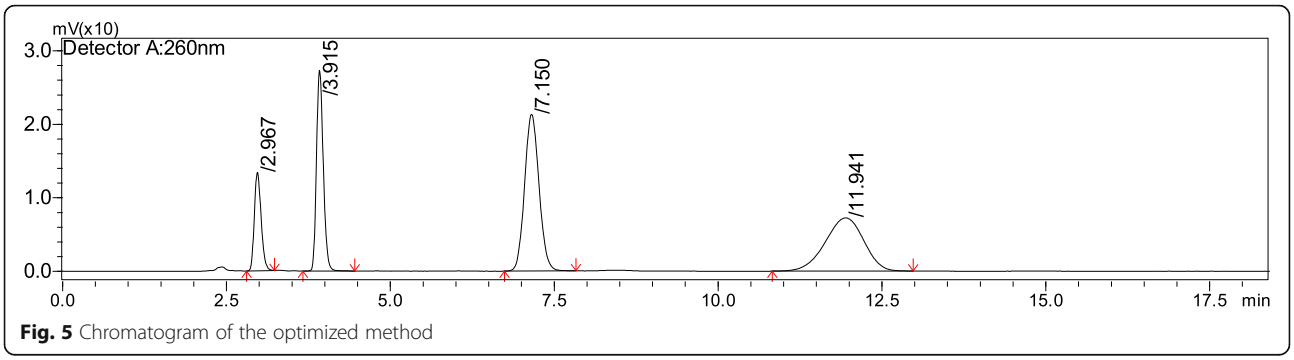

Fig. 5 Chromatogram of the optimized method

Table 6 Chromatographic results for the optimized method

S.No	Name of the drug	Retention time	Area	HETP	Resolution	Tailing factor	Theoretical plates
1	Daclatasvir	2.96	96,858	42.97	–	1.26	3940.49
2	Sofosbuvir	3.91	195,757	24.21	4.7	1.24	6194.80
3	Velpatasvir	7.15	326,395	30.61	10.6	1.10	4899.29
4	Ledipasvir	11.94	297,377	77.56	6.4	1.09	2115.98

Table 7 Results of the method validation

Parameter		Results of sofosbuvir	Results of velpatasivir	Results of ledipasivir	Results of daclatasivir
Linearity	Linearity range	60–360 μg/mL	40–140 μg/mL	40–140 μg/mL	20–120 μg/mL
	Correlation coefficient (R^2)	0.999	0.999	0.998	0.998
	Regression equation	y = 3276x – 44,583	y = 5226x – 95,804	y = 5089x – 90,670	y = 3500x + 20,863
Sensitivity	LOD (μg/ml)	1.389	0.082	0.622	0.772
	LOQ (μg/ml)	4.167	0.246	1.866	2.316
Precision (% RSD of peak area)	Intra-day precision	0.303	0.953	0.893	0.836
	Inter-day precision	0.566	0.99	0.92	0.956
Robustness (% RSD of peak area)	Flow rate (± 0.1 ml/min)	0.63	0.336	0.163	1.13
	pH (± 0.2)	0.896	0.406	0.16	1.32
	Organic phase (± 1%)	0.62	0.34	0.15	1.2
	Wavelength (± 2 nm)	0.623	0.37	0.303	1.53
System suitability	Retention time (min)	3.91	7.15	11.94	2.96
	Theoretical plate number	6194.8	4899.29	2113.99	3940.49
	Tailing factor	1.24	1.10	1.13	1.26
	Resolution	4.7	10.6	6.4	-

Table 8 Assay of the marketed formulations

Formulation	Sofosbuvir				Velpatasvir/ledipasvir/daclatasvir			
	Label claim (mg)	Amount found (mg) (A.M ± SD)	% assay	% RSD	Label claim (mg)	Amount found (mg) (A.M ± SD)	% assay	% RSD
ECLUPSA (SOF + VEL)	400	410.8 ± 1.23	102.7	0.29	100	102.3 ± 0.59	100.2	0.57
HARVONI (SOF + LED)	400	408 ± 1.49	102	0.36	90	89.09 ± 0.25	98.9	0.28
DARVONI (SOF + DAC)	400	409.2 ± 1.56	102.3	0.38	60	61.05 ± 0.45	101.75	0.73

A.M arithmetic mean, *SD* standard deviation, *%RSD* relative standard deviation

were attempted. A satisfactory separation of the drugs was obtained by XTERRA RP18 (250 × 4.6 mm, 5 μ) column eluted with acetonitrile and potassium dihydrogen phosphate buffer of pH 5.0 in the ratio of 50: 50 % v/v by isocratic elution pattern at a flow rate of 1.0 ml/ min with a detection wavelength of 260 nm for sofosbuvir, velpatasvir, ledipasvir, and daclatasvir. of trial methods were as shown in Fig. 5, and its peak results were reported in Table 6.

Method validation

The method was optimized and validated according to ICH Q2 (R1) [32] guidelines. A linear response was

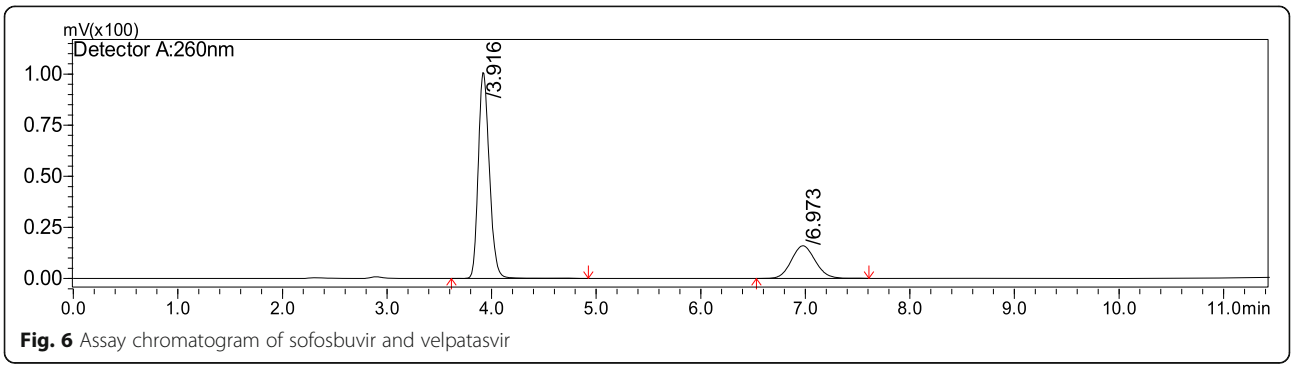

Fig. 6 Assay chromatogram of sofosbuvir and velpatasvir

The injection volume of 20 μl at 25 °C temperature afforded the best separation of these analytes. The retention time was found to be 2.96 min for daclatasvir, 3.91 min for sofosbuvir, 7.15 min for velpatasvir, and 11.94 min for ledipasvir. The optimized method conditions were shown in Table 5. The chromatograms willful over the examined concentration range of 60–360 μg/ml for sofosbuvir, 40–140 μg/ml for velpatasvir, 40–140 μg/ml for ledipasvir, and 20–120 μg/ml for daclatasvir. Chromatograms of blank and placebo had a dearth of the peak at the retention time of title drugs indicated the specificity of the method. The accuracy of

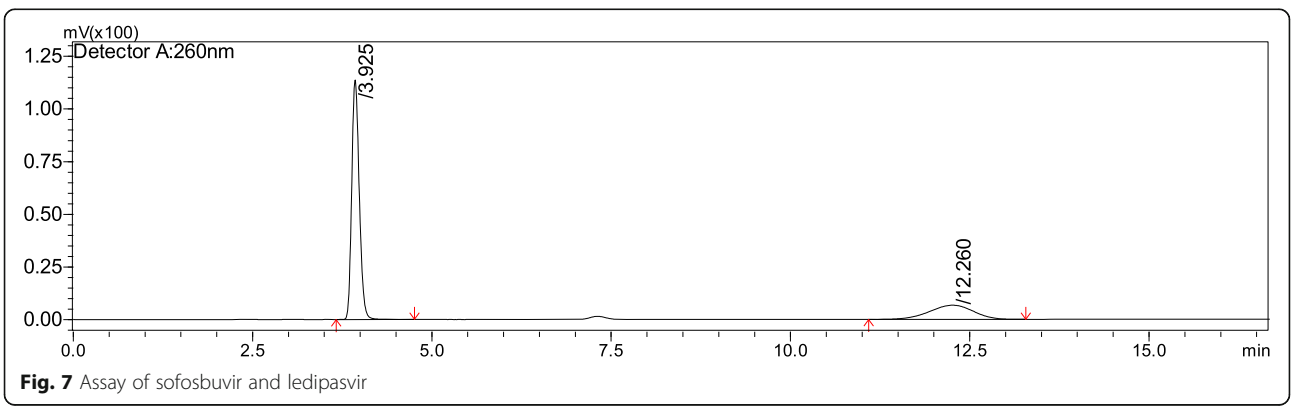

Fig. 7 Assay of sofosbuvir and ledipasvir

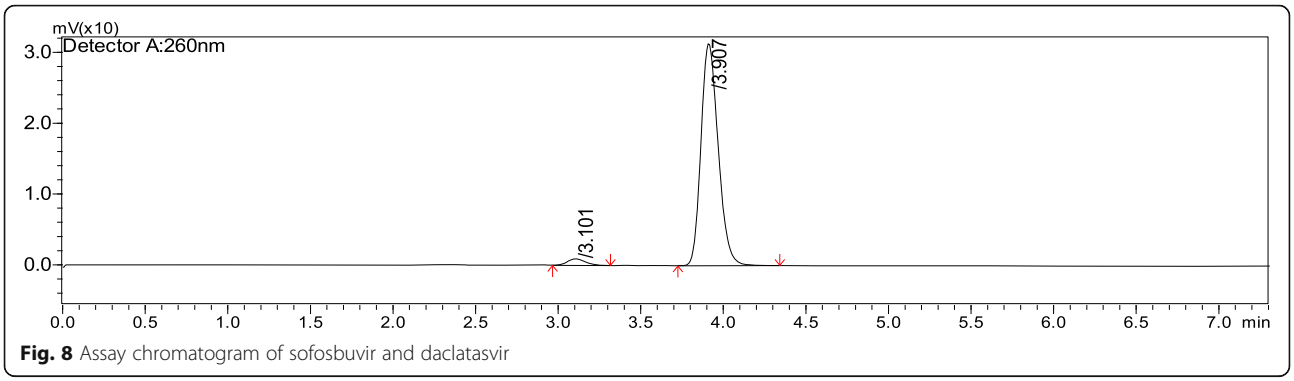

Fig. 8 Assay chromatogram of sofosbuvir and daclatasvir

the method was validated by recovery studies and was found to be significant under specification limits, with afforded recovery of 98.8–101% for sofosbuvir, 99–102% for velpatasvir, 97–105% for ledipasvir, and 96–105% for daclatasvir. The % RSD values for intra-day and inter-day precision were less than 2.0, which endorsed the good repeatability of the proposed method. Predicted response of optimized method parameters in the robust process (MODR of contour plot) was verified experimentally, and no significant change in retention time, peak area, and tailing factor and plate count by deliberate variations in method parameters was observed. The results of the system suitability test and validation were represented in Table 7.

Assay of marketed dosage form

The proposed method was evaluated by the assay of commercially available tablet dosage forms. The results obtained were compared with the corresponding labelled amounts and reported in Table 8. The % assay results of sofosbuvir and velpatasvir in EPCLUSA was found to be 102.7% and 100.2%, sofosbuvir and ledipasvir in HARVONI found to be 102% and 98.9%, and % assay of sofosbuvir and daclatasvir in DARVONI was found to be 102% and 101.7%, respectively. The %RSD was less than 2, which indicates the accuracy of the proposed method. The assay chromatograms of respective drugs were shown in Figs. 6, 7, and 8, respectively.

Discussion

The rising need for multi-drug anti-viral therapy shapes novel challenges in analytical research. The complexity in separating the drugs with different physico-chemical properties from the quaternary mixtures and applying them to the pharmacokinetic study was eased by AQbD. The research mainly focused on developing a method that has very little run time in just 16 experimental trials, which makes it more sustainable. The statistical outcomes also granted excellent linearity and specificity. The merits of the method such as low LOQ, high resolution, and good theoretical plate number compared to some peer journals [21–24] suggest its use for in vitro and in vivo characterization of novel formulation and in quality control. The central composite design suggested an increase in flow rate to obtain high resolution, and acidic pH in separating the basic components is good due to their more likeliness for protonation/ionization. The method catches its credibility by showing a resolution (> 2), tailing factor (0.9–1.2), capacity factor (1–10), number of theoretical plates (> 2000), and very good HETP.

Conclusion

To our present knowledge no trials have been made yet to assay all four anti-viral drugs in a mixture. So, the robust, reproducible, sensitive, specific, inexpensive analytical method for simultaneous assessment of anti-viral drugs in co-formulated dosage forms has been developed using a central composite design. The proposed single (low consumption of solvent) analytical method can be employed for routine quality control analysis and pharmacokinetics studies of combined anti-viral drugs of sofosbuvir/velpatasvir, sofosbuvir/ledipasvir, and sofosbuvir/daclatasvir simultaneously.

Abbreviations

AQbD: Analytical quality by design; HPLC: High-performance liquid chromatography; MODR: Method operable design region; ICH: International Conference on Harmonization; RSD: Relative standard deviation; SOF: Sofosbuvir; VEL: Velpatasvir; LED: Ledipasvir; DAC: Daclatasvir; ATP: Analytical target profile; CMP: Critical method parameters; SS: Sum of squares; CQA: Critical quality attributes; HETP: Height equivalent to a theoretical plate

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Authors' contributions

Conception and design of the study were done by RTJ under the guidance of PDA and PRA. RTJ played a prominent role in the acquisition of the data. Analysis and/or interpretation of the data were taken care of by RTJ and PDA. Drafting of the manuscript was the job taken by RTJ. PDA revised the manuscript critically for important intellectual content. The version of the manuscript to be published was read and approved by all the contributing authors.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
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