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Response Surface Optimization for Simultanous Estimation of Sofosbuvir and Velpatasvir in Human Plasma Sample

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ABSTRACT

An efficient column friendly, buffer free, highly sensitive, cost effective RP-HPLC method was developed by considering the criticality of different method parameters on analytical attributes like tailing factor, resolution and retention time in preliminary risk analysis and screening designs. The Pareto analysis of screening design highlighted the need for optimization of resolution and its influencers (capacity factor and theoretical plates) for both the drugs to imbibe quality in the method. The suggested method of optimization design was developed using ZODIAC C18 ODS (250 mm × 4.6 mm, 5 µm) column in isocratic mode using mobile phase acetonitrile : methanol : water in the ratio of 60:10:30 at a flow rate of 0.8 mL/min and UV detection wavelength of 262 nm. The retention times of drugs were found to be 3.488 min for sofosbuvir, 5.387 min for velpatasvir. The linear regression analysis data for the calibration plots showed good linear relationship with r^2=0.997 for sofosbuvir, r^2=0.988 for velpatasvir, in the working concentration range of 1000-5000 ng/mL, 250-1250 ng/mL respectively. The AQbD devised method was applied for quantification of drugs in plasma and validated as suggested in ICH M10 guidelines.

Keywords: AQbD, HPLC, Sofosbuvir, Velpatasvir, Central composite design, Taguchi design, Fractional factorial design, Bioanalytical.

INTRODUCTION

The global statistics data released by WHO made a conjecture that nearly 71 million people worldwide were infected by chronic *Hepatitis* C and in most of them disease has been progressed to cirrhosis and hepatocellular carcinoma.[1] Though there was 95% chance of reducing deaths and disease

progression by antiviral therapy, lack of access to proper diagnostics and treatment kept the numbers rising through decades. Given the consequences there was imperative need for sustained research activity on development of novel antiviral entities, targeted therapies and combination therapy.[2]

EPCLUSA was single dose effective treatment regimen for genotypes 1-6 of *Hepatitis C virus*. It is a combination of nucleotide analogue sofosbuvir which functions as NS5B polymerase inhibitor and velpatasvir which inhibits NS5A protein of HCV. The directly acting antiviral drug sofosbuvir was reported to be most effective sustainable therapeutic drug individually or in combination with other drugs like velpatasvir, ledipasvir, daclatasvir, and ribavarin. The 12 week therapy of the combination sofosbuvir 400 mg and velpatasvir 100 mg have implicated good therapeutic index in *Hepatitis* C infected patients with or without compensated cirrhosis.[3] The serious side effect bradycardia when consumed with anti arrhythmic drugs and decrease in solubility with rise in pH for velpatasvir suggested conceptual and sequential risk ranking based parameter choosing for method development was need of an hour. The spectral overlap of sofusbuvir and velpatasvir was also suggested as considerable risk according to peers.[4]

Literature revealed there is only one QbD method for simultaneous analysis of four antiviral drugs sofosbuvir, velpatasvir, ledipasvir and daclatasvir by RP-HPLC using sigma tech software for design of experiments.[5] There is no multi factor optimized bioanalytical method for simultaneous estimation of sofosbuvir and velpatasvir till date. Very few chromatographic methods have been reported in literature for estimation of sofosbuvir alone or in combination with velpatasvir and other drugs in plasma by HPLC[6,7,8], UPLC[9–13], LC-MS/MS[14],HPTLC.[15] A few spectrophotometric methods and densitometric methods were also reported.[11,16,17] Most of the chromatographic methods developed by peers used buffer of pH 1.8-3.5[18,13] which affects the shelf life of the column, and some RP–HPLC methods used pH 6-7 in the composition of mobile phase.[6,10,19–21] The mobile phase was composed of organic modifier acetonitrile 30-80% by some peers,[6,13,17,21,22,24] whereas methanol 75% was used in one article[25] and methanol and acetonitrile 40:40 was used in one method development study.[19,26] The wavelength was varied from 240-285 nm and column temperature, flow rate was maintained at 30^0C, 1 mL/min in all the methods. The methods were reported using C8 and C18 columns with length varying from 250mm to 150mm.From consideration of all literature method variations of one factor at a time multi factor response optimized method development was initiated. The intricate study of analytical attribute data from literature indicated the methods with good resolution ranging from 3.7-10.66 have been developed but the capacity factor in some methods was just near the acceptable region instead of ideal value.[5] The tailing of velpatasvir was also recorded 1.75 with the method in literature,[21] So the present method states the analytical target profile as to develop a method which is sustainable, easily transferable without any further requirement for revalidation and should be capable of quantifying drugs in nanograms. The method also proposed to show its suitability parameters good compared to other developed methods by peers.[27]

MATERIALS AND METHODS

Materials

The HPLC grade acetonitrile, water and methanol were purchased from Merck limited Mumbai and the drugs sofosbuvir and velpatasvir were procured as gift samples from Hetero labs limited Hyderabad. The Systronics PC based double beam spectrophotometer 2202 with 1 cm matched quartz cells was used to scan both drugs. The method development was executed in Shimadzu prominence liquid chromatograph with SPD-20A UV–VIS detector and Symmetry ODS C18 (4.6 x 250mm, 5 μm) column. Screening was performed on Prontosil C 8 (250 x 4.6 mm, 5 μm), Prontosil C 18 (150 x 4.6 mm, 5 μm), Supelco discovery C8 (150 x 4.6 mm, 5 μm) columns.The output signal was monitored and integrated using Lab solutions software. Schimadzu electronic balance, Digital ultrasonic cleaner ultra sonicator, Cyber scan pH meter, Fischer scientific nylon membrane filter (0.45 μm, 47mm) and C24 Remi cooling centrifuge were operated in the method development and validation. Design expert version 13.0 (Stat ease Mineapolis, USA) software loaded in HP laptop with Intel i7 processor was used for screening and optimization of critical method.

Analytical target profile

Analytical target is to develop a highly sensitive, column stable, robust, high resolute RP-HPLC method for estimation of antiviral drugs sofosbuvir and velpatasvir from plasma without use of buffer to improve shelf life of the column. The multifactor optimization by QbD improves the suitability of the method by decreasing the need for revalidation.[28]

Critical analytical attributes

Thorough review of methods developed by the peers and initial risk assessment by Ishikawa fish bone cause and effect diagram suggested the resolution, retention time of drugs and tailing factors as the important analytical attributes that accounts good separation of analytes from plasma in less run time with high extracting efficiency.

Design of experiments

The design of experiments software (DESIGN EXPERT TRIAL VERSION 13) was downloaded from STAT EASE to know the effect of the critical method variables on the quality of the method developed.[29]

Screening design

Taguchi experimental design was used to screen seven factors in eight experimental trials at two different levels and given in Table 1. The experiments were performed on the HPLC instrument to prioritize the critical variables in order of their effectiveness on the analytical attributes. The Pareto charts indexed the effect of each factor and deliberately explained the interaction effect of each factor on other method variables. The seven factors screened were column length(150mm,250mm), column chemistry (C8,C18), pH(3,7), type of organic modifier (acetonitrile, methanol), %organic phase (40,60), wavelength (240,260), flow rate (0.8,1.2). The responses resolution, retention time of drugs and tailing factors were considered critical attributes to compare the efficiency of the developed method with literature methods. The screening factors with a critical t value were further optimized using response surface analysis.

Response surface optimization

Twenty experimental trials consisting of 6 axial points,8 factorial points and 6 centre points with α= 0.6 modeled central composite design trials were performed randomly to mitigate bias in results on HPLC–U V chromatographic system connected to Symmetry ODS C18 column (4.6 x 250mm, 5μm) at a column temperature 30^{0}C, and a wavelength of 262nm. The retention time and tailing factors are within limits for all the trials of screening design, so resolution and its effectors were given importance in response surface optimization. Three critical method variables organic phase(60-70), flow rate(0.8-1.2), ratio of organic modifiers(3:1-6:1) were optimized to study quality of method developed by knowing their effect on analytical attributes resolution and its effectors like capacity factor and theoretical plates of sofosbuvir and velpatasvir. The organic modifier ratio elucidated the effect of polarity index on resolution.

Table 1: Taguchi design for screening variables

Run	Variable						
	1	2	3	4	5	6	7
	A	B	C	D	E	F	G
1	-1	+1	+1	+1	+1	-1	-1
2	+1	+1	-1	+1	-1	-1	+1
3	+1	-1	+1	-1	+1	-1	+1
4	+1	-1	+1	+1	-1	+1	-1
5	-1	-1	-1	+1	+1	+1	+1
6	-1	+1	+1	-1	-1	+1	+1
7	-1	-1	-1	-1	-1	-1	-1
8	+1	+1	-1	-1	+1	+1	-1

Data Analysis

Of the various designs like linear 2FI, quadratic and cubic, best-fit model was selected based on parameters, like PRESS (predicted error sum of squares), R^2 (adjusted, predicted), adequate precision, % coefficient of variation (CV), and degrees of freedom for pure error, lack of fit analysis. The statistical analysis of experimental designs was executed in Design expert software to study significance of variables on attributes. The importance of variables was identified using ANOVA (Fisher's statistical test) for the analysis the variance. The correlation coefficients (R^2) were estimated for responses utilizing multiple regression analysis. The Perturbation plots indicated amount of interaction among variables, 2D contour plots and 3D response surface plots were employed for detecting range of variation favoring the desirable values for analytical attributes.

Numerical and graphical screening of optimized data was done taking derringer's desirability index (~1) as the basis for the criteria set for analytical attributes. The numerical optimization

picked the points with desirable values for method variables to obtain results for analytical attributes (resolution, capacity factor and theoretical plates) as set in the goals before. The graphical optimization created method operable design region to work with, without the need for revalidation. The points in desirable region were experimentally performed during robustness study to determine accountable error in experimental results using the formula.

$$\% \text{ accountable error} = \frac{(\text{lab value-predicted})}{\text{Predicted}} \times 100$$

Preparation of mobile phase

The HPLC grade acetonitrile, methanol and water were kept in ultrasonic water bath for 5 minutes to degas, filtered through 0.45 μm nylon membrane under vaccum and mixed in ratio of 60:10:30 respectively. The mobile phase was used as diluent for preparation of standard and sample solutions.

Preparation of standard solution

Accurately weighed and transferred 10 mg of each standard drug in to two different 10 mL of volumetric flasks, add about 7 mL of diluent, sonicated to dissolve, diluted to the mark with diluent and mixed well which produces a concentration of 1000 μg/mL. The above solutions were further diluted to obtain solutions in the concentration range of 1000-3000 ng/mL for SOF and 250-1250 ng/mL for velpatasvir.

Sample preparation

A simple two step liquid-liquid extraction (LLE) procedure was carried out for the extraction of sofosbuvir and velpatasvir from plasma samples. To a series of 500 μL of drug solutions prepared, 200 μL of plasma and 600 μL acetonitrile were added and mixed for 2 min for de proteination and centrifuged at 5000 rpm for 20 minute. The organic layer was separated and from this required amount was taken and diluted to 10 mL with methanol. This solution was then injected into HPLC.

Optimum chromatographic conditions

The separation of two drugs with efficient resolution, sensitivity was obtained through RP-HPLC method devised with symmetry ODS C18 column set at a column temp of 30^{0}C and wavelength of 262nm. The mobile phase optimized was acetonitrile: methanol: water in the ratio of 60:10:30 at a flow rate of 0.8 mL/minute. The runtime was 10 min and injection volume was 20 μL.

Method validation

The predicted parameter developed method using optimum chromatographic conditions was validated for system suitability, specificity, linearity, precision, accuracy, sensitivity and robustness according to standard guidelines confronted by ICH M10.

Specificity

The specificity of the method was studied by observing the spectra of sample and standard solutions at different concentrations to notice absence of interference. The chromatogram plotted at LLOQ was also included in sensitivity studies.[22]

Linearity and range

The five point calibration curve was prepared on single day for the method developed. The results obtained were subjected to linear regression analysis equipping least square method to determine equation for line. The extracted plasma samples containing 1000–5000 ng/mL of sofosbuvir and 250-1250 ng/mL of velpatasvir were injected each time into the column and the corresponding chromatograms were obtained. From these chromatograms retention times and the area under the curve of the drug sample was compared to that of the reference standard for each dilution. A relevant calibration curve was constructed with concentration on x-axis and area under the curve on y-axis.

Accuracy

Accuracy of the method was determined by recovery experiments. The known amount of standard drug sofosbuvir and velpatasvir, (50%, 100%, and 150%) sample is spiked and percentage recovery values were calculated.

Precision

The repeatability of the method was examined at single intermediate level by injecting the solution consisting of sofosbuvir and velpatasvir in to the HPLC system for two consecutive days (intraday and inter-day) respectively. The %RSD values of the results corresponding to the peak area and retention time were calculated.

Robustness verification

The optimised HPLC conditions set for this method have been slightly modified for samples of sofosbuvir, velpatasvir and the data was compared with optimization design data. The small changes include the change in flow rate, percentage organic phase and wavelength. The solution was injected by making small changes in flow rate (±0.1 mL/min), % organic phase (±5%) and wavelength (±2nm).

Stability Studies

Three of LQC and HQC were stored at the intended storage temperature for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 to 24 h under the same conditions. The freeze–thaw cycle were repeated two more times and then analyzed on the third cycle. For short term stability studies three aliquots of LQC and HQC were thawed at room temperature and kept at this temperature for 22 h and analyzed. The long-term stability was determined by storing three aliquots of LQC and HQC under the same conditions as the study samples for 22 days. The stability of stock solutions of the drug was evaluated at room temperature for 6 hours.

RESULTS AND DISCUSSION

Preliminary studies

IR spectral studies, UV spectra recording and melting point determination aided in authenticating the drugs. The individual drugs were scanned in the range of 200-400nm and the spectra was overlaid to determine λ_{max} for further experimentation. The wavelength 260-262nm was observed as the best choice as both drugs showed good absorbance in this range.

Screening studies

Highly economical and reasonably efficient fractional factorial design was executed to screen multiple factors in 8 experimental trials at two levels without any blocks in completely randomized model. Through ANOVA and regression analysis the significance of variables on attributes were studied and those variables which have ($p<0.05$) were considered significant. The half normal plots and pareto charts of resolution (SOF/VEL), retention time of SOF, VEL, tailing factor of SOF and VEL indicated importance of choosing organic phase, organic modifier and flow rate to improve method quality. The Pareto charts and half normal plots indicated order of hierarchy in variables and positive and negative influence of variables explained by color blocking them blue and orange. The factors like column length, column chemistry, wavelength, injection volume and column temperature were fixed based on desirable index in numerical optimization of taguchi design. The following inferences were drawn from screening analysis.

1. Flow rate of mobile phase (F) has shown to affect peak symmetry and stationary- mobile phase interactions, making it a critical variable. The %organic component (B) is demonstrated as critical in resolution and drug retention times of the drug. The organic phase has affirmative effect on retention times (low retention time) and decreasing effect on resolution.
2. The long length columns 250mm was preferred over 150mm as they increased the resolution and decreased the tailing. The C18 column is the choice after screening all factors as the column improved resolution and selectivity by changing the interaction pattern with drugs having polar surface area 159(Sofusbuvir) and 193(Velpatasvir).
3. Column chemistry (D) has minor impact on resolution. The ODS column (C18) was fixed, because it offered better analyte selectivity and peak shape than in C8 column.
4. The incorporation of acetonitrile in organic phase decreased the retention time of sofusbuvir to greater extent and velpatasvir to less extent. It improved resolution but considerable tailing was also noted with 70% acetonitrile.
5. The injection volume showed good effect on tailing of drug but the effect on all other attributes was nullified. As designated by risk assessment via Pareto analysis, %organic phase, organic modifier and flow rate were identified ($p<0.05$) as critical method variables (CMVs) and chosen for subsequent chromatographic optimization studies. The change in retention time and tailing was within considerable limits, so resolution was opted for optimization.

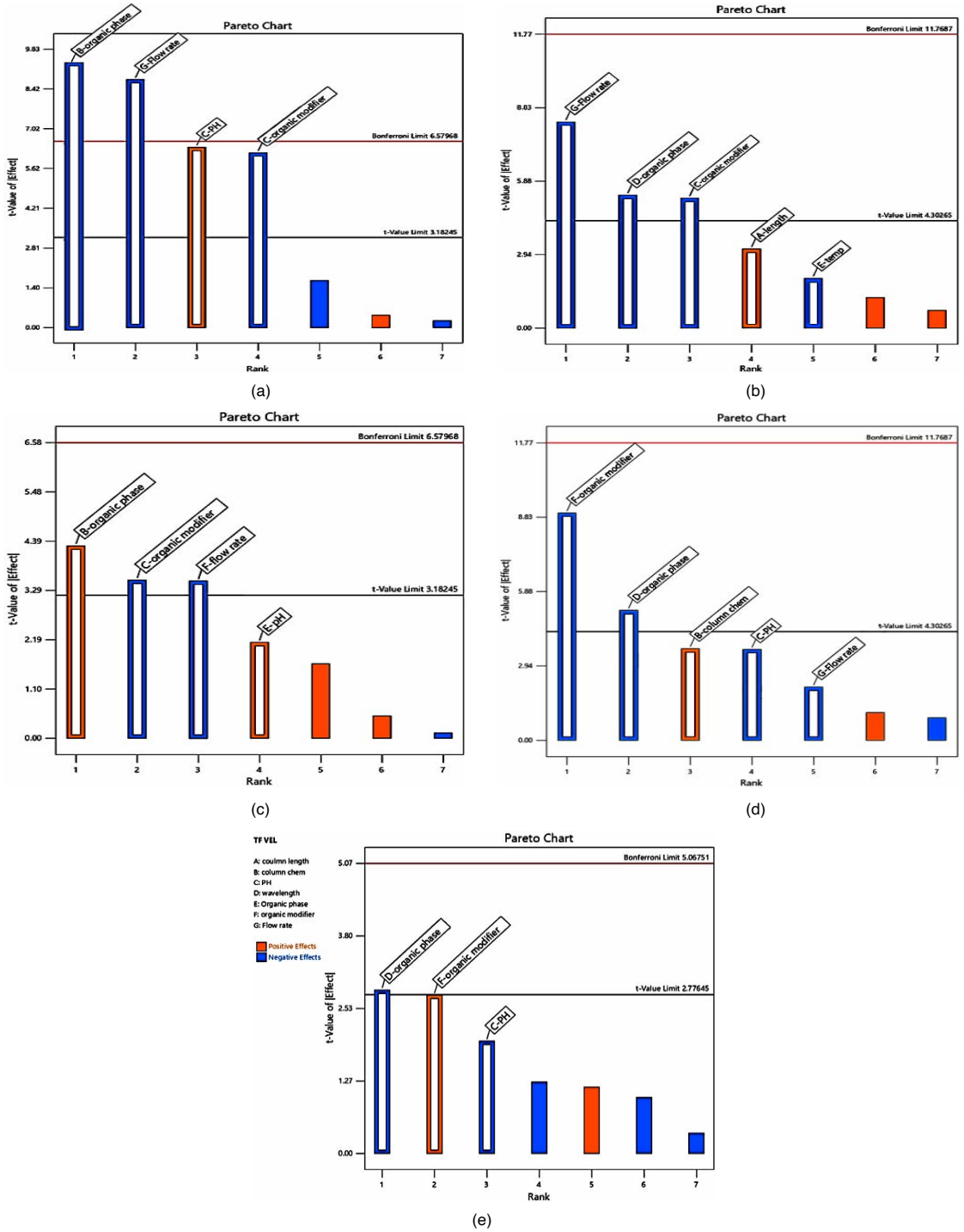


Fig. 1. Pareto ranking analysis 1a) retention time of sofosbuvir 1b) retention time of velpatasvir 1c) resolution of Sofosbuvir and Velpatasvir 1d) tailing of sofosbuvir 1e) tailing of velpatasvir

AQbD approach method optimization

The most crucial variables in screening were optimized employing highly efficient central composite design. The effect of dependent variables organic phase, modifier ratio and flow rate on independent variables (resolution, capacity factor, theoretical plates) was explored by central composite design with 20 experimental trials. The experimental

results of 20 trials obtained were given in Table 2 and were optimized. The linear model was suitable for analysis of data related to resolution and capacity factor whereas quadratic model was the method of choice for theoretical plates of both drugs. The ANOVA and regression analysis data was abridged in Table 3.The regression equations of all the analytical attributes represented as

$$Y1(Resolution) = 8.467-0.866743X_1+1.46405X_2 -2.83602X_3$$

$$Y2\ (K_1') = 2.3993-0.438602X_1-0.279079X_2 -0.72779X_3$$

$$Y3\ (K_1') = 3.97525-0.570117X_1-0.126773X_2 -0.977227X_3$$

$$Y4\ (TP_1) = 1629.1-271.239X_1 +103.715X_2 -859.613X_3-7.625\ X1X_2+50.875X1X_3-10.125X2X_3 +210.539X_1^2-34.6691X_2^2+226.456X_3^2$$

$$Y5(Tp2) = 2138.32-222.992X_1+112.208X_2 -833.939X_3-43.625X1X_2 +82.375X1X_3 +29.875X_2X_3 +229.177X_1^2-74.7522X_2^2 +217.347X_3^2$$

Table 2: Central Composite design

Std	Run	Factor 1 A:organic phase	Factor 2 B:organic modifier ratio	Factor 3 C:flow rate	Response 1 resolution	Response 2 CF SOF	Response 3 CF VEL	Response 4 TP SOF min	Response 5 TP VEL
5	1	60	4	1.2	4.1	2.629	3.827	1290	1650
3	2	60	6	0.8	14.1	3.317	5.742	3460	3890
9	3	57.3768	5	1	10	3.08	4.77	2493	2920
4	4	70	6	0.8	13.84	2.48	4.279	2593	2926
19	5	65	5	1	9.6	2.45	4.135	1493	1990
15	6	65	5	1	8.3	2.307	3.92	1790	2234
6	7	70	4	1.2	3.2	1.623	2.763	657	1190
7	8	60	6	1.2	7.1	1.895	3.445	1383	1890
8	9	70	6	1.2	6.2	1.064	2.374	966	1475
12	10	65	6.52465	1	9.3	1.795	3.445	1760	2278
20	11	65	5	1	8.5	2.467	4.087	1420	1860
2	12	70	4	0.8	8.9	2.946	4.576	2490	2980
11	13	65	3.47535	1	6.1	2.59	3.894	1480	1879
18	14	65	5	1	9.6	1.865	3.546	1550	1967
13	15	65	5	0.69507	10.4	3.875	5.578	3390	3875
10	16	72.6232	5	1	5.8	1.805	3.067	1887	2650
1	17	60	4	0.8	11.4	3.876	5.593	3080	3550
16	18	65	5	1	8.1	2.005	3.601	1745	2345
14	19	65	5	1.30493	5	1.384	2.574	1064	1640
17	20	65	5	1	9.8	2.533	4.289	1680	2280

Table 3: ANOVA and regression analysis data

Respon se	Y1	Y2	Y3	Y4	Y5
Sum of Squares	138.53	10.12	16.39	1.148E+07	1.080E+07
Df	3	3	3	9	9
Mean Square	46.12	3.37	5.46	1.27E+06	1.200E+06
F-value	35.34	67.15	99.97	43.41	21.89
p-value	<0.00 01	<0.000 1	<0.00 01	<0.0001	<0.00 01
SD	1.14	0.2241	0.2338	171.42	234.14
Mean	8.47	2.40	3.98	1883.55	2373.45
% CV	13.49	9.34	5.88	9.10	9.86
r^2	0.8689	0.9264	0.9494	0.9750	0.9517
Adj R^2	0.8443	0.9126	0.9399	0.9526	0.9082
Pred R^2	0.7834	0.8983	0.9267	0.8613	0.7397
Adeq precisio n	20.2261	28.8447	32.0226	21.6247	15.3594
PRESS	34.50	1.11	1.21	1.633E+06	2.954E+06

R^2-correlation coefficient; PRESS-error of sum of squares;%CV- coefficient of variation;F value-Fischer's t-test value; p value –significance value(p <0.05 indicates significance)

The perturbation plot given in Fig. 2 explains the effect of analytical attributes (responses) in deviating the value of dependent variables from their standard values. The amount of curvature, deviation compared to other factors and slope value quantifies the effect of response and effect of interactions. The perturbation plot suggested highest curvature is associated with theoretical plate's number of sofosbuvir and velpatasvir. The 2D and 3D plots shown in Fig. 3, 4 plotted keeping one variable constant and modifying other two variables within the range, confront the desirable area by contouring blue to red indicating Derringer's desirability (0-1).The curvilinear graph of theoretical plates indicates quadratic relationship between attributes and method variables. The flow rate

change(X_3) was the major contributor having negative influence on all responses, so minimum flow rate considering pressure on column was parameter of choice. The organic phase showed decreasing trend on resolution, capacity factor and theoretical plates. The organic phase with highest percentage of acetonitrile depicted decrease in theoretical plate number whereas interaction with flow rate has positive influence(X_1X_3) on theoretical plates of sofosbuvir and velpatasvir. The organic phase percentage in mid range with high ratio of acetonitrile were favored variations for theoretical plates. The highest effect of organic modifier ratio was seen on resolution, capacity factor of sofosbuvir followed by theoretical plates of velpatasvir. The organic modifier ratio has positive influence on resolution, theoretical plates and showed negative lineage on capacity factor of sofosbuvir and velpatasvir. From the optimization analysis the flow rate 0.8-1mL/mim, organic phase (65-70%) and ratio (6:1-5.5:1) was considered commendable.

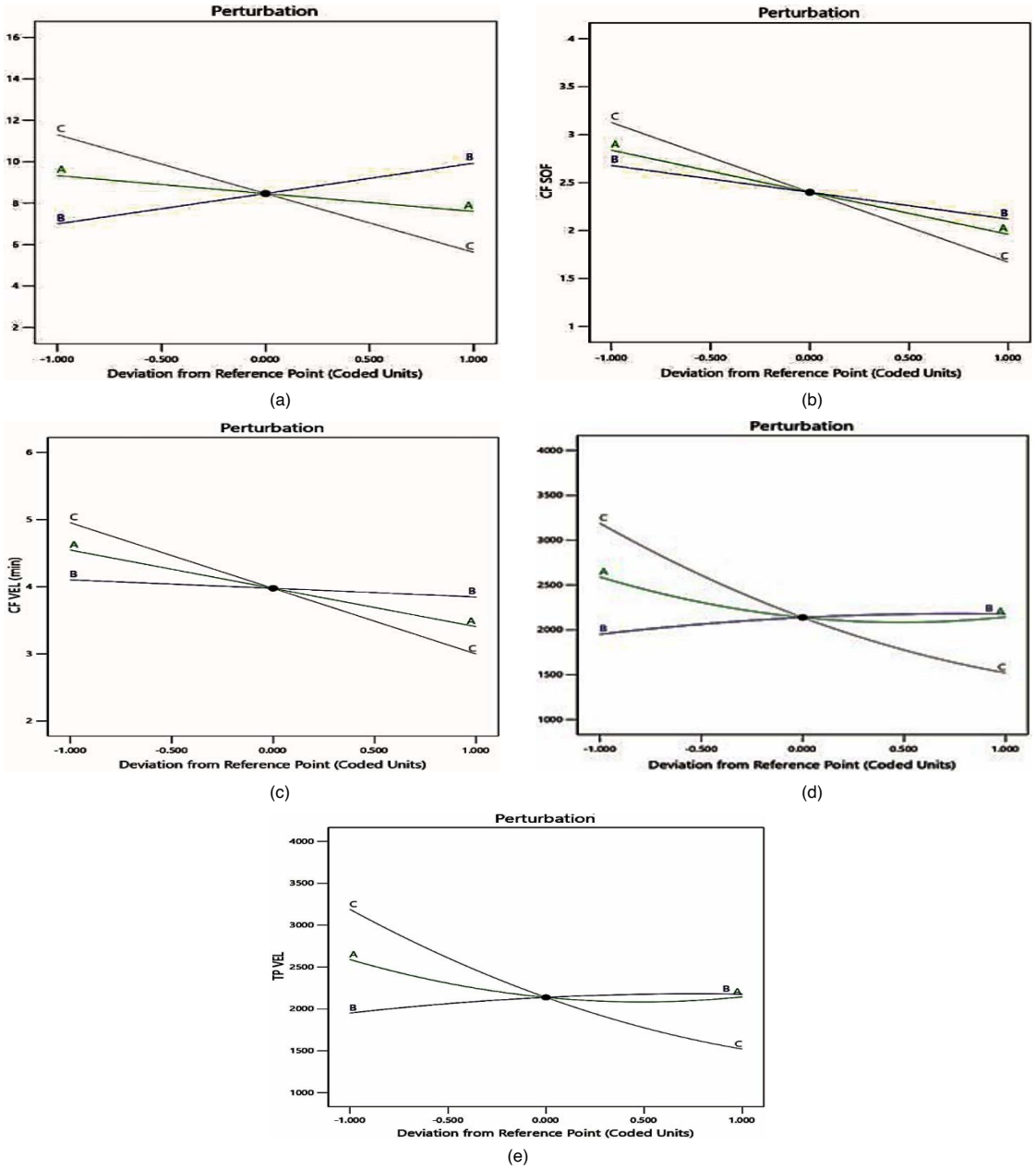


Fig. 2. Perturbation plots 2a)resolution 2b)capacity factor SOF 2c) capacity factor VEL 2d) theoretical plates SOF 2e) theoretical plates VEL

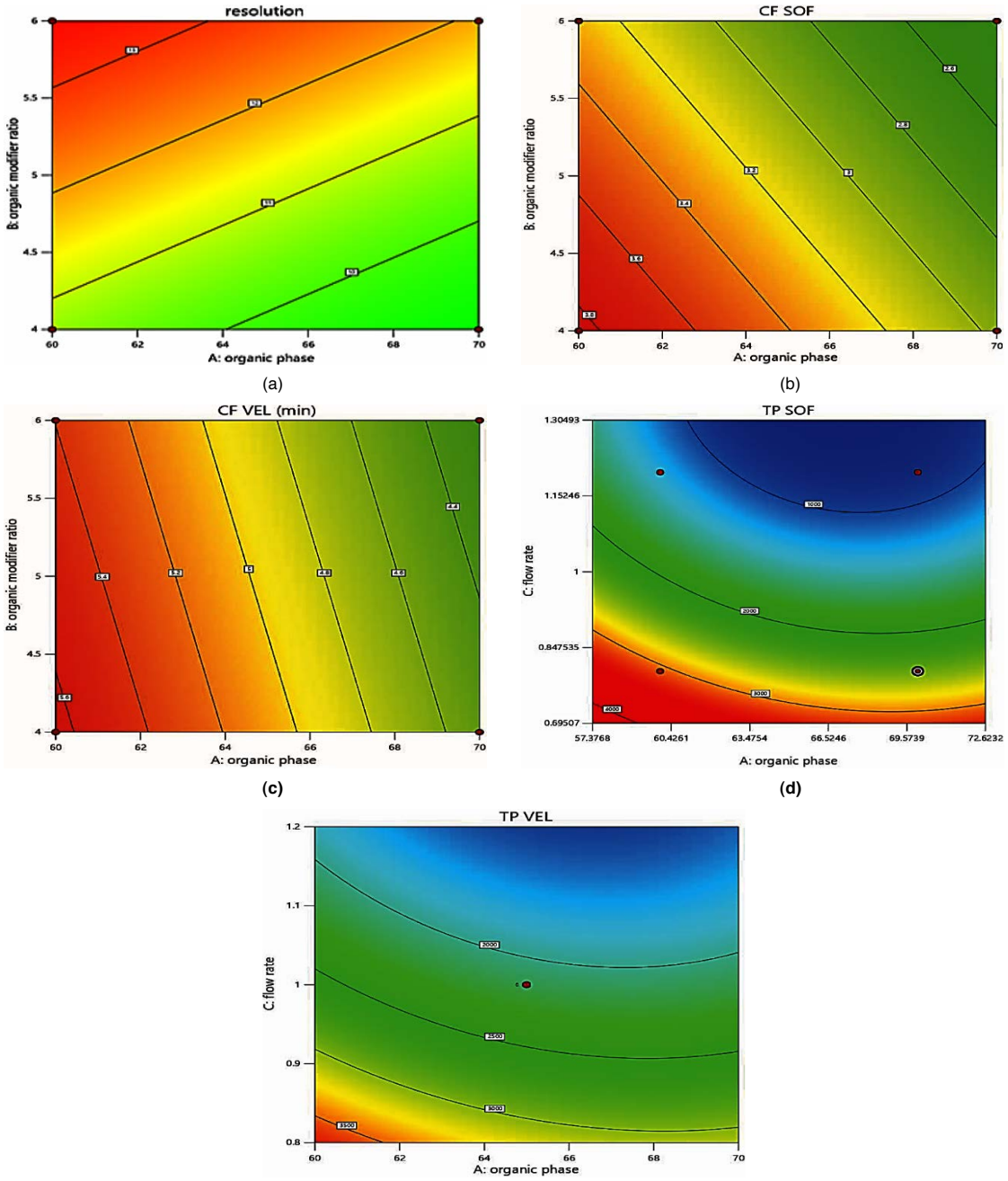


Fig. 3. 2D plots 2a) resolution 2b) capacity factor SOF c) capacity factor VEL d) theoretical plates SOF e) theoretical plates VEL

Numerical optimization was executed giving the criteria as maximization for resolution, capacity factor within range (1.5-5) and maximization of theoretical plates with minimum number stated as 2000. The four star importance was applied to resolution and other factors were subjected to three star optimization. The five solutions of fifteen options obtained in point prediction table having maximum desirability was opted for experimentation and the % bias in the result was calculated during robustness study and tabulated in Table 4 The %bias was less than 6% indicating the aptness of the design application in method development. The graphical optimization indicated range of feasible variations in overlay plot along with most desirable point flagged on it given in Figure 5.

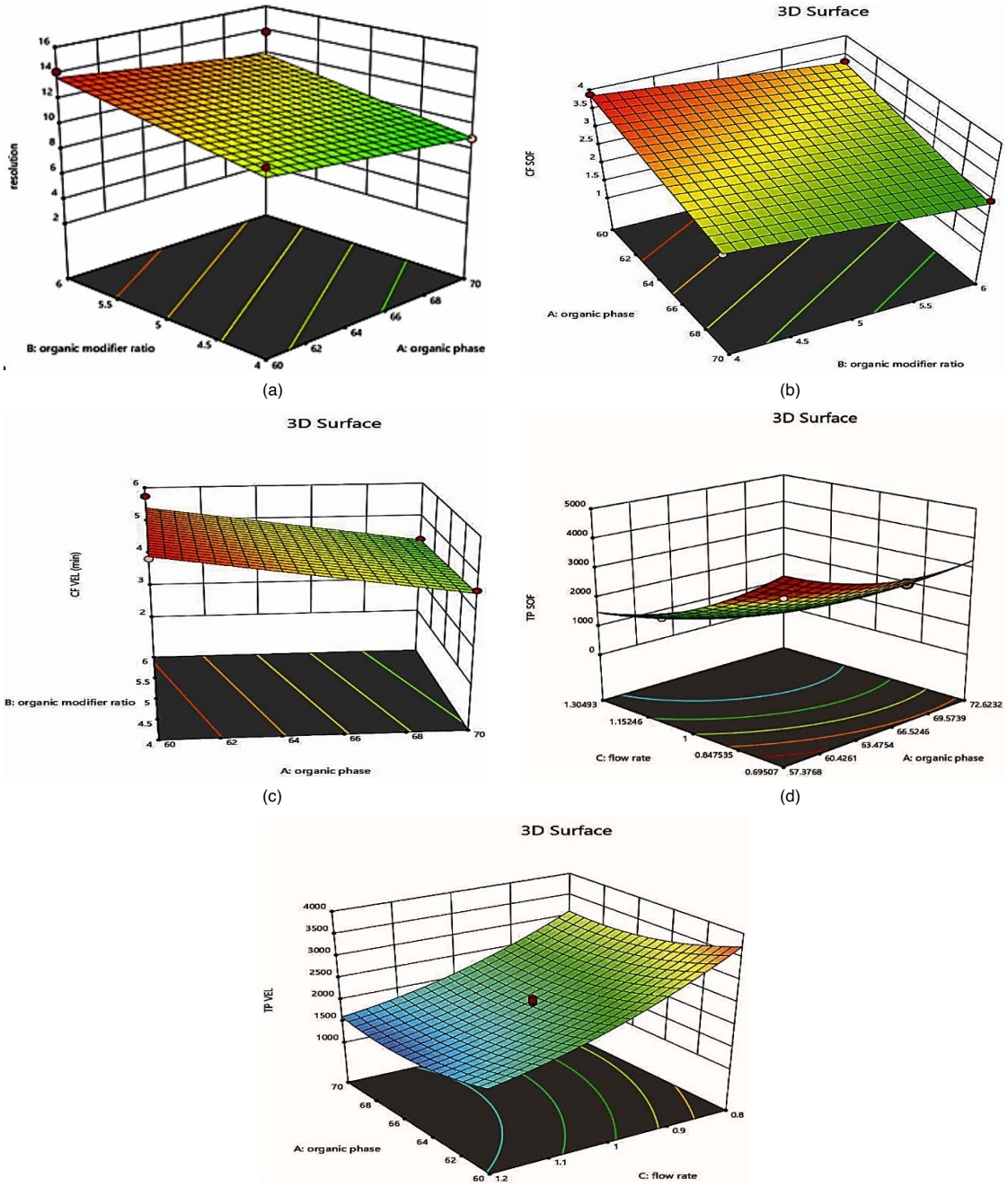
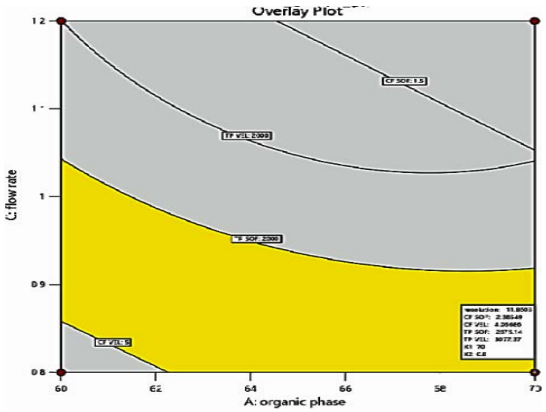


Fig. 4. 3D plots 4a) resolution 4b)capacity factor SOF 4c) theoretical plates SOF 4d) capacity factor VEL 4e) theoretical plates VEL

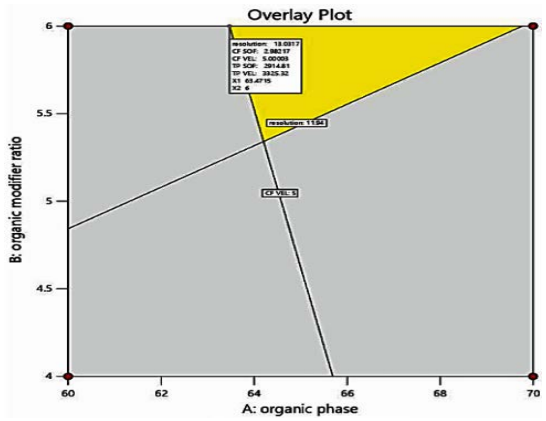
System suitability

The separation of the bulk drugs in plasma was obtained by Symmetry ODS C18 (250 x 4.6mm, 5μ) with acetonitrile, methanol and water in the ratio of (60:10:30 %v/v) by Isocratic elution mode at a flow rate of 0.8mL/min with a detection wavelength of 262nm for sofosbuvir, velpatasvir. The injection volume of 20 μL at 30°C temperature afforded highly resolved peaks (13.84) with retention time of 3.488 for sofusbuvir and 5.387for velpatasvir. The theoretical plate number was 2630 for sofosbuvir and 3036 for velpatasvir which ratifies the suitability of the method. The tailing factor of both drugs was within limits, 1.37 for SOF and 1.12 for VEL. The chromatograms of

the optimized method showed no interference and were in good acceptable signal to noise ratio. The chromatogram was shown in Figure 6.



(a)



(b)

Fig. 5. Overlay plot taking a) keeping acetonitrile: methanol-6:1 varying other two parameters b) flow rate constant 0.8mL/min varying other two parameters

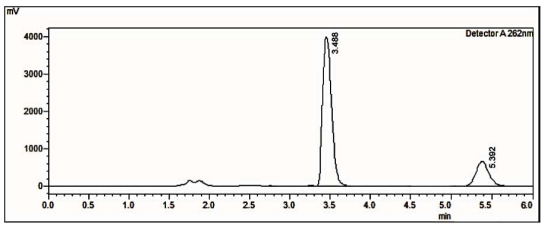


Fig. 6. optimized chromatogram

Method validation

The ICH M10 guidelines were strictly employed in validation of the method.[30] The method indexed good sensitivity in the linearity range of 1000-3000ng/mL for sofosbuvir and 250-1250ng/mL for velpatasvir. The chromatograms of blank and plasma showed dearth of peak at the retention time of analyte drugs. The accuracy of the method was validated at LLOQ and three other concentrations and was found to be significant under specification limits with afforded recovery 99.66-100.44% for sofosbuvir, 100.18-100.39 %for velpatasvir. The repeatability of the executed method was tested by precision studies at LLOQ and two other concentrations in six replicates. The %RSD values for intra-day and inter-day study were less than 2.0 endorsing applicability of the method. The robustness was verified by altering %organic phase, flow rate, wavelength and temperature for points in experimental design. The robustness testing suggested no significant variation in quality of method developed. The results of validation were represented in Table 4. The stability study chromatogram of short term, long term, freeze and thaw cycle and stock solution showed no traces of degradant peaks and %RSD of peak area and retention time was found to be less than 2. The values from experimental data were also in good confirmation with design of space results with an error less than 6% indicating predictability.

Table 4: Validation data for analysis of drugs

Parameter	Linearity range	Results of Sofosbuvir 1000-6000 ng/mL	Results of Velpatasvir 250-1250 ng/mL
Linearity	Correlation co-efficient (R^2)	0.997	0.988
	Regression equation	Y = 16.75X	Y= 15.29X
Sensitivity	LOD (μg/mL)	0.05	0.1146
	LOQ (μg/mL)	0.1629	0.3475
Precision (% RSD of peak area)	Intra-day precision 10	0.22	0.82
	30	0.68	0.37
	50	0.79	0.44
	Inter-day precision 10	0.50	0.45
	30	0.58	0.36
	50	0.88	0.83
Accuracy (% RSD of recovery)	10 μg/mL	0.16	1.4
	30 μg/mL	0.85	0.36
	50 μg/mL	0.77	0.9
Freeze thaw stability	10 μg/mL	0.18	1.24
	30 μg/mL	0.51	0.38
	50 μg/mL	0.84	0.90
Short term stability	10 μg/mL	0.27	1.02
	30 μg/mL	0.97	0.33
	50 μg/mL	0.43	0.40
	10 μg/mL	0.24	1.42
Long term stability	30 μg/mL	0.94	0.33
	50 μg/mL	0.25	0.90
% bias(mean change)	1000ng/mlSOF+250ng/ml VEL	2.3	4.5

DISCUSSION

Identifying the precedence of multifactor analysis over one factor at a time analysis, the multi factor optimized method was developed and validated. After a keen view on literature models and primary risk analysis the factors needed to be screened to develop quality in method was attributed. The Pareto ranking and half normal plot study of taguchi design illustrated the criticality ranking of different parameters on attributes like resolution, retention time and tailing factor. The parameters above the level of significance (t=3.108) were optimized by highly efficient central composite design. The screening design criticality indexed variables like %organic phase, organic modifier ratio, and flow rate were response surface optimized for resolution and its influencers. The perturbation plots evinced interaction between the terms and response, whereas the 2D, 3D plots and coefficient values in regression equation suggested the range of variables favoring desirable response. The numerical and graphical optimization was performed by registering system suitable ranges in criteria and solution obtained were filtered to choose five best responses with maximum derringer's desirability index. The results of experimental and predicted data were compared to improve the significance of AQbD application.

CONCLUSION

The highly sensitive RP-HPLC method to quantify the drugs sofosbuvir and velpatasvir in plasma was developed without use of any buffer for secondary equilibrium. The application of Quality by design principles in development of method reduced the risk of revalidation within the zone of analytical target profile. The method showed very good resolution of 13.84 with all system suitable parameters being within range. There were very few bionalytical methods in literature and till date there is no bioanalytical method developed by multi factor optimization. Multiple points in the design space can be operated to develop reliable chromatographic method. The method can be extended for analysis of clinical samples as a part of pharmacokinetics or equivalence studies.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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