ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

Quality-by-design approach to selective stability indicating RP-HPLC method development and validation for estimation of Sofosbuvir in bulk drug

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Article History:

Received on: 21.08.2017
Revised on: 15.12.2017
Accepted on: 03.02.2018

Keywords:

Stability indicating assay
RP-HPLC
Sofosbuvir
QbD
Validation

ABSTRACT

By utilizing Quality by design approach a simple, robust and selective Reverse phase liquid chromatography (RP-HPLC) method was developed for the estimation of Sofosbuvir in API. Sofosbuvir is a nucleotide Hepatitis C Virus (HCV) NS5B polymerase inhibitor that has antiviral activity. Analyte target profile of Sofosbuvir was constructed and CAA's were identified. LC analysis was performed on Agilent LC 1200 equipped with a photodiode array detector (PDA) at 261 nm. Initial method was developed on an Inersil ODS (250 mm x 4.6, 5 micron) 65: 35 Methanol and Water at 261nm witha flow rate of 1 mL/min. The degradation studies of Sofosbuvir were carried out under the stress conditions of hydrolysis (acid, base and neutral), oxidation, photolytic and thermal as per ICH guidelines. The alkali hydrolysis shows more critical impurities which were well resolved from pure drug with the application of DoE. Three method variables were selected and their interaction were studied through central composite design and two method responses like retention time of drug and resolution between degradants and active drug were studied. Based on DoE, the optimized method was developed using 60:40 of Methanol and Water, at pH 4 with a flow rate of 1.2 mL/min which produce satisfactory retention time and resolution. This method has been validated and shown to be linear, accurate, robust, precise and specific.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v9i2.1439

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INTRODUCTION

Sofosbuvir is a new drug candidate for Hepatitis C Virus(HVC) treatment, with the chemical name L-Alanine, N-[[P(S),2′R]-2′-deoxy-2′-fluoro-2′-methyl-P-phenyl-5′-uridylyl]-, 1-methylethyl ester (Harmeet Kaur Bhatia et al 2014). Sofosbuvir is a NS5B polymerase inhibitor of nucleotide Hepatitis C Virus (HCV) that has genotypic antiviral activity and a high genetic barrier to resistance (Gillian M. Keating, 2014). The molecular formula of Sofosbuvir is $C_{22}H_{29}FN_3O_9P$ [fig.1] and molecular weight is 529.458 g/mol. It is a white to off-white crystalline solid.

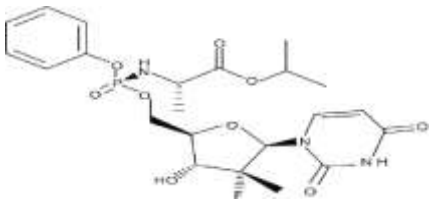

Figure 1: Structure of Sofosbuvir

Upon oral administration, Sofosbuvir is converted into the active tri-phosphate nucleotide after metabolized to 2'-deoxy-2'-alpha-fluoro-beta-C-methyluridine-5'-monophosphate which, inhibits the NS5B polymerase and preventing viral replication. The HCV NS5B protein is essential for the replication of the viral HCV RNA genome which is an RNA-dependent RNA polymerase.

International Conference on Harmonization (ICH) Q8 (R1) guideline defines QbD as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management." (ICH Q8)

Analytical sciences are considered as an integral part of pharmaceutical development. Implementing the QbD approach to analytical method development, popularly termed as "Analytical QbD (AQbD)". AQbD helps in development of a robust and cost effective analytical method which is applicable throughout the lifecycle of the product, to facilitate the regulatory flexibility in analytical method. It means the freedom to change method parameters within a method's design space, referred to as the Method Operable Design Region (MODR) (Ramalingam Peraman, 2015).

In the literature there are many RP-HPLC methods (Sandya Rani & Devanna, 2017; Panchumarthy-Ravisankar, 2017; Ravikumar Vejendla, 2016; Uppalapati Jyothi, Parimi Umadevi, 2017), UV methods (R. M. Nemade, 2017), RP-HPLC and UV method (Suryaprakash Y. Rai, 2017), Bioanalytical (Madhavi & Prameela Rani, 2017), stability indicating method of combined dosage form (Sarath Nalla & Seshagiri Rao, 2017) and single dosage form (Nemade, 2017), Forced degradation study by LC-MS MS (Nebsen & EmanElzanfaly, 2016) and dissolution study (Mohamed El-Kassem M Hassouna, 2017) has been reported on Sofosbuvir but QbD based stability indicating assay methods on Sofosbuvir has not been reported anywhere. In the reported stability indicating methods also they were not focused on risk assessment & method transferability. So, there is need to develop liquid chromatography method for stability indicating assay method of Sofosbuvir by using Quality by Design approach to understand method variable that critically affect on method performance and providing the design space to overcome method failure.

The primary objective of study was to develop more robust, cost effective, selective, economic and regulatory flexible RP-HPLC method for stability indicating assay employing QbD approach.

MATERIALS AND METHODS

Reagents and chemicals

The active pharmaceutical ingredient was received as gift sample from Aurobindo pharmaceuticals, Hyderabad, India. HPLC-Grade methanol (Me-OH) was purchased from Merck, Darmstadt, Germany.

Instrumentation and chromatographic conditions

Chromatographic separations were carried out using Agilent LC system (LC-1200 series), consisting of a binary pump, a Rheodyne injector with a 20µL loop and a photodiode array detector (DAD) at 261 nm. Separation was accomplished on an Inersil ODS (250 mm x 4.6, 5 micron) with a flow rate of 1 mL/min. The column temperature was maintained at room temperature. By using Ezchrome Elite software which is resident in a Pentium computer (Digital Equipment), the output signal were processed. The prepared mobile phases were filtered through a membrane filter (0.45 µ) and degassed using sonicator prior to use.

Table 1: Quality Target Product Profile for Sofosbuvir

QTTP Element	Target	Criticality
Dosage form	API and Degradation products	Not applicable
Appearance	White to half white powder	Not applicable
Impurity percentage	0.05%	Critical
Method type	Reverse phase	To get good retention time for drug
Instrument type	Binary pump	For effective mixing, efficient separation
Stability indicating assay	API and Degradation products	To indicate the stability of product under various stress conditions

Defining Quality Target Product Profile (QTPP)

It is a prospective summary of quality characteristics of a drug product taking into account together with the attributes affecting method performance. Retention time, theoretical plates, resolution between drug and degradants formed during forced degradation were selected a Critical Analytical Attributes (CAA) (Table 1).

Preparation of standard solutions

A stock solution of Sofosbuvir (100 mg/ml) was prepared by dissolving the drug in Methanol–Water (1:1, v/v) diluents.

Forced degradation study

To conduct the forced degradation study, pure API of Sofosbuvir was subjected to various stress conditions. As Sofosbuvir is freely soluble in mobile phase, Sofosbuvir were dissolved small amount in mobile phase later diluted with in 0.1M aqueous Hydrochloric acid and 0.1M Sodium Hydroxide for 25 hrs and 1hr 40 min, respectively, while neutral hydrolysis was carried out in water for 72 hrs. All the hydrolytic studies were carried out at room temperature. By taking 0.3% v/v of H_2O_2 (30% w/v) at room temperature the Oxidative degradation was carried out for 50 hrs. By exposing a thin layer of the solid drug in a Petri-dish as well as solutions of the drug in water to sunlight for 10 hrs the Photolytic studies were done. The thermal degradation was carried out by spreading the Sofosbuvir powder to about 1mm thickness in a Petri dish and kept at 60°C in the oven for 72 hrs. After the degradation, these solutions were neutralized and diluted with mobile phase was subjected to analysis.

Table 2: Critical Method Parameters

Condition	Criticality
HPLC Instrument	Control
Column	Control
Detector	Control
Temperature of column	Control
Vendor for Mobile phase	Control
Glass wares	Control
API standard	Control
pH of mobile phase	Variable X_1
Percentage of aqueous in Mobile phase	Variable X_2
Flow rate of Mobile phase	Variable X_3

Risk Assessment

Risk assessment identifies the Critical Method Variables (CMV), the parameters that impact the ATP. "It is systematic process for the assessment, control, communication and review of risks to the quality across the product lifecycle" (Table 2).

Design of Experiment

Based on the risk assessment the influencing method parameters were identified which affect the method performance like accuracy, precision, robustness, specificity. The X_1, X_2, X_3 were considered as Critical Method Parameters in Experimental design to bring out the rationale relationship between the input variables (CMP) and method response (Retention time and Resolution).

RESULTS AND DISCUSSIONS

Selection of Chromatographic Conditions

A detection wavelength of 261 nm was selected from the full range UV spectral for 100 µg/ml of Sofosbuvir. The gradient run was assessed using mobile phase-A: Methanol and mobile phase B: Water 80:20 v/v to 60:40 v/v) on C8 and C18 columns.

The optimized chromatographic condition, system suitable parameters were listed in table 3. The optimized chromatogram was shown in fig 2.

Forced degradation

Stress degradation studies of Sofosbuvir were carried out under hydrolysis (acid, base and neutral), oxidation, photolytic and thermal conditions as per stated in procedure. The Chromatograms of forced degradation studies of Sofosbuvir were show in fig 3.

From the degradation studies the oxidative degradation shows zero percentage of degradation, thermal degradations were not shown presence any impurities, where acid hydrolysis was shown only one impurity. The alkali hydrolysis chromatogram shows relatively more number of impurities and also two critical impurities likely to co-elute with the active drug.

Hence, the alkali degraded sample has been taken into consideration for method optimization under Quality by Design approach.

Method Optimization using Design of Experiment

Based on the risk assessment the influencing method parameters were identified. The X_1, X_2,X_3 (Table 2) were considered as Critical Method Parameters in Experimental design to bring out the rationale relationship between the input variables (CMP) and method response (Retention time and Resolution). Three variables are selected X1, X2 and X3 i.e. pH, aqueous percentage and flow rate. In this method, the method response Retention time (Y1) is considered as indicator of robustness of the method which affect the method performances and other method response Resolution (Y2, Y3, Y4) between impurities and active drug were taken. Primarily 2^3factorial design were selected where three variables in two levels which was given in the table 4. After the processing it showed non linear so Central composite design were selected to evaluate the interaction between selected variables and runs were conducted as per listed in table 5 and shown in figure 4. Numerical and graphical optimization was carried out to get an optimal "Analytical Design Space" region.

Table 3: Optimized Chromatographic Conditions

Optimized Chromatographic Conditions	
Instrument	Agilent LC system (LC-1200 series)
Injector	Rheodyne injector
Pump	Binary pump
Detector	photodiode array detector
Column	Inersil ODS (250 mm x 4.6, 5 micron)
Temperature	Room temperature
Detection wavelength	261 nm
Mobile phase	Methanol: Water (65:35)
pH of Mobile phase	4
Flow rate	1 mL/min.
Injection volume	20 µL loop
Concentration of standard	25 µg/Ml
System Suitability Parameters	
Retention time	7.007 min
Peak area	1061943
Theoretical plate	3383
Asymmetry	0.83859

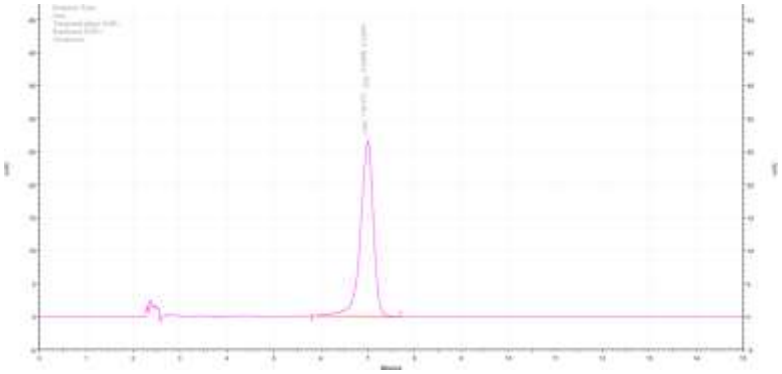

Figure 2: Optimized Chromatogram of Sofosbuvir

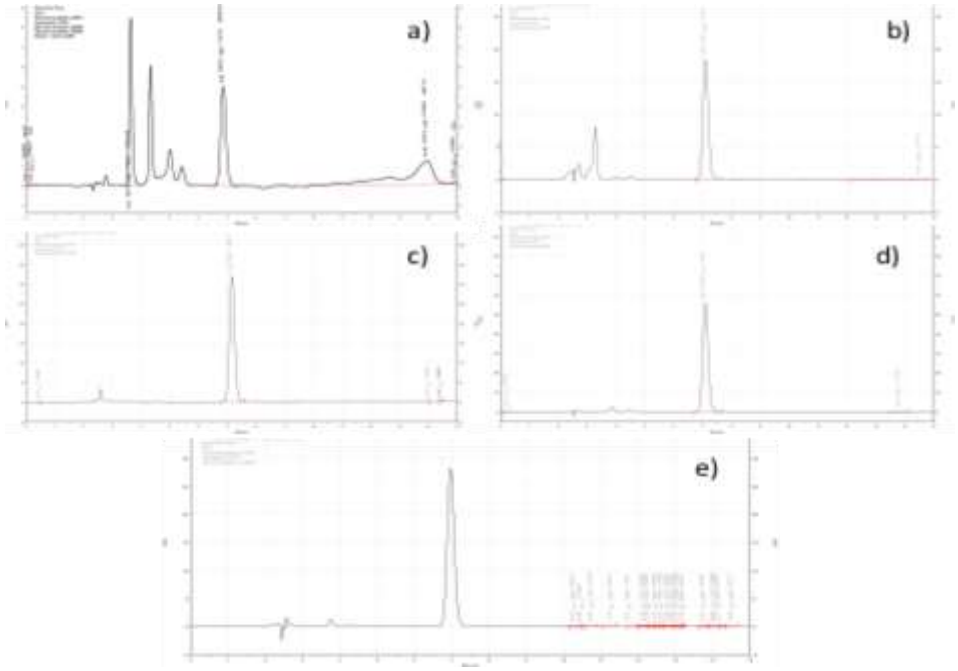

Figure 3: Forced degradation chromatograms of Sofosbuvir under different stress conditions a) Alkali hydrolysis: 0.1M NaOH1hr40min; b) Acid Hydrolysis: 0.1 M HCl 25hrs; c) 3 % v/v H_2O_2: 50 hrs; d) Sun light – 10 hrs; e) Thermal: 60°C for 72 hrs

Table 4: Design of Experiments- Levels of Variables

Name of variables	Units	-2 levels	-1level	0 level	+1level	+2 levels
pH of aqueous phase	-	3	3.5	4	4.5	5
Percentage of aqueous phase	%	25	30	35	40	45
Flow rate	mL/min	0.8	0.9	1	1.1	1.2

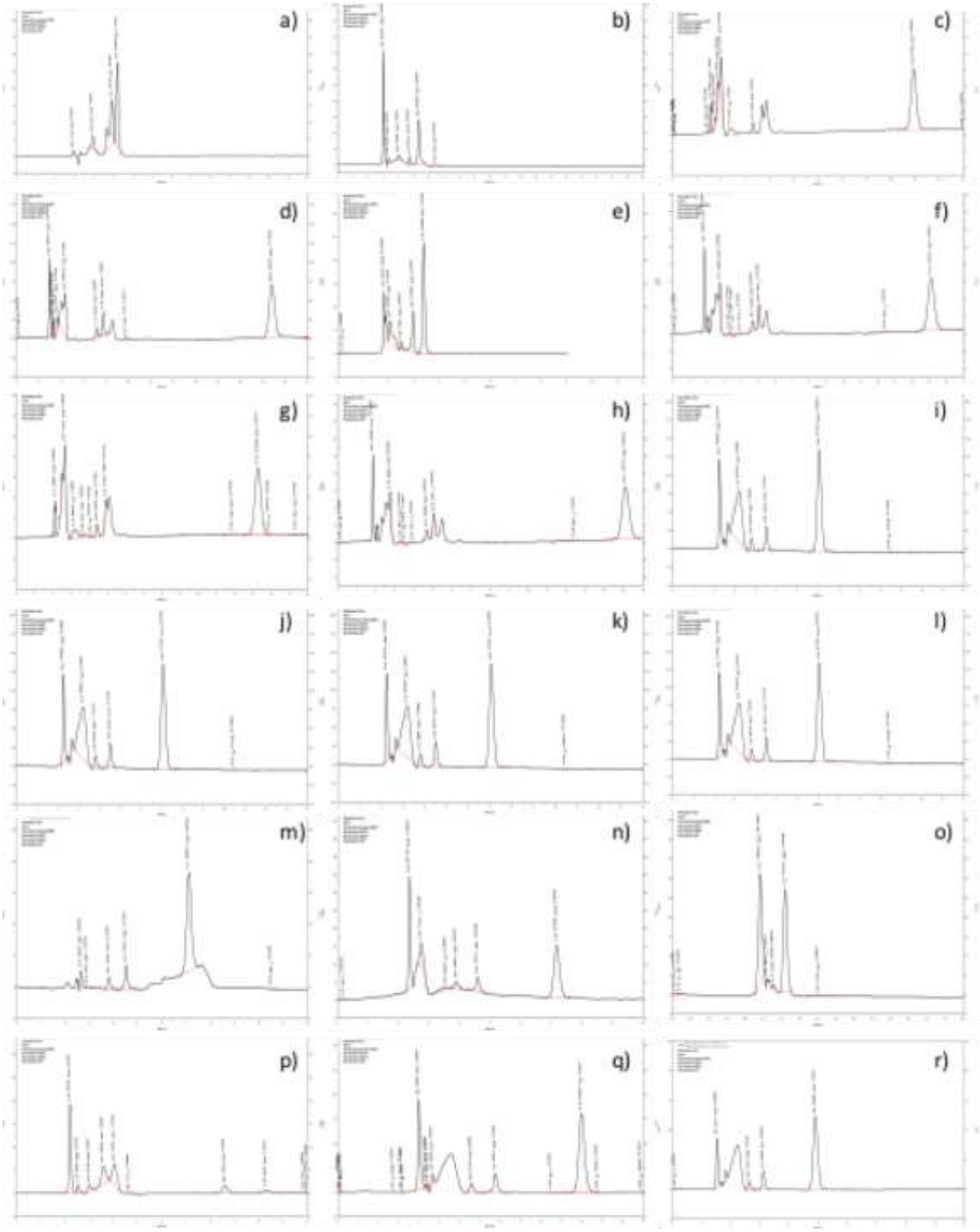

Figure 4: Trail Chromatograms as per Design of Experimental design a) Combination I- 25: 75 pH3 FR(0.8); b) Combination X1-25:75 pH5 FR(0.8); c) Combination X2 - 45: 55 pH 3 FR (0.8); d) Combination X1X2-45: 55 pH5 FR(0.8); e) Combination X3- 25 : 75 pH3 FR (1.2); f) Combination X1X3- 25 : 75 pH5 FR (1.2); g) Combination X2X3- 45: 55 pH3 FR(1.2); h) Combination X1X2X3- 45 : 55 pH5 FR(1.2); i) 35: 65 pH4 FR (1.0) MID POINT1; j) V1 35:65 pH4 FR(1.0) MID POINT2; k) 35: 65 pH4 FR(1.0) MID POINT3; l) 35 :65 pH4 FR(1.0) MID POINT4; m) 35: 65 pH2 FR (1.0); n) 35: 65 pH6 FR (1.0); o) 15: 85 pH4 FR(1.0); p) 55: 45 pH4 FR(1.0); q) 35: 65 pH4 FR (0.6); r) 35: 65 pH4 FR (1.4)

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Table 5: Central composite design for screening of method parameters

Combinations	pH of aqueous phase	Percentage of aqueous phase	Flow rate	Retention time	Resolution	Resolution	Resolution
	(X1)	(X2)	(X3)	(Y1)	(Y2)	(Y3)	(Y4)
I	3	25	0.8	5.19	0.0000	4.33396	3.11551
X1	5	25	0.8	5.32	2.28382	3.59074	4.068
X2	3	45	0.8	19.89	27.12701	3.27376	3.32642
X1X2	5	45	0.8	22.82	16.65302	6.59343	1.85627
X3	3	25	1.2	3.33	3.72796	2.77123	1.13975
X1X3	5	25	1.2	3.38	18.81828	2.66057	3.27847
X2X3	3	45	1.2	13.27	3.45665	1.456851	3.39871
X1X2X3	5	45	1.2	15.11	14.3385	8.14743	5.65218
MID POINT	4.0	35.0	1.0	7.05	0.0000	8.4534	3.11336
MID POINT	4.0	35.0	1.0	7.0	0.0000	8.4534	3.12336
MID POINT	4.0	35.0	1.0	7.1	0.0000	8.3034	3.11336
MID POINT	4.0	35.0	1.0	7.25	0.0000	8.3234	3.12336
AVG MIDPOINT	4.0	35.0	1.0	7.1	0.0000	8.3584	3.1184
CCD	2.0	35.0	1.0	7.11	0.0000	2.40261	3.41566
CCD	6.0	35.0	1.0	7.18	0.0000	0.86331	2.239998
CCD	4.0	15.0	1.0	3.1	22.71089	0.0000	0.0000
CCD	4.0	55.0	1.0	4.03	0.0000	2.26199	0.0000
CCD	4.0	35.0	0.6	12.0	0.0000	10.10577	3.62592
CCD	4.0	35.0	1.4	4.91	10.42683	7.45185	2.8682

Table 6: Analysis of Observed Data for Experimental Design

Combinations	Name of variables	Coefficient Value	F-Value Y1	SS Ratio
b0	-	11.0388	0.0	-
b1	pH	0.6187	32.7171	0.7228%
b2	%aqueous phase	6.7338	3875.5609	85.6167%
b12	pH & %aqueous phase	0.5738	28.1407	0.6217%
b3	Flow rate	-2.2662	438.9455	9.6969%
b13	pH and flow rate	-0.1462	1.8269	0.0404%
b23	aqueous phase & Flow rate	-1.3163	148.0894	3.2715%
b123	pH, aqueous phase & flow rate	-0.1263	1.3634	0.0301%

Error variance:0.0117, SD :0.108, 95% Confident level of curvature effect -4.1492 to 13.7283, Non-linear; F Std value at 0.1p:5.54, F Std value at 0.05p:10.1, F Std value at 0.01p:34.1

Table 7: The interaction analysis of variable's coefficient value for Y1, Y2, Y3, Y4

Combination	Y1	Y2	Y3	Y4
B0	11.0756	6.1303	13.7481	10.8876
B1	0.3181	0.3094	0.117	0.1624
B2	3.4831	0.528	3.6496	3.3669
B3	-2.0194	0.1702	-1.4649	-1.2278
B12	0.5738	0.5738	0.5736	0.5738
B13	-0.1462	-0.1462	-0.1462	-0.1462
B23	-1.3163	-1.3163	-1.3163	-1.3163
B11	-0.4057	-0.7663	-2.3551	-1.0438
B22	-1.3307	2.0726	-2.4806	-1.7507
B33	-0.1582	0.5371	-0.5686	-0.939

Statistical Analysis of Method Response

Statistical analysis of the data obtained from factor screening and method optimization were performed using the Sigma Tech Software table 6. The following polynomial equation behavior of the system was explained by the

$$Y=b0+b1X1+b2X2+b3X3+b12X1X2+b23X2X3+b13X1X3 + b123X1X2X3 \ldots.\text{Eqn. 1.}$$

Where, Y is the response (retention timeY1 and resolutionY2, Y3, Y4), b0 is the intercept, b1, b2, b3 are the regression coefficients of variables for pHX1, % aqueous phaseX2 and flow rate X3 re-

spectively. b12, b23, b13 are the regression coefficients for two factor interactions between variables and b123 is the coefficient for three factor interaction between X1X2X3.

Y1 (Retention Time of Drug)

From the interaction analysis the SS ratio describes the % factor that affects retention time. From the analysis (table6) the variable pH shows least effect on retention time followed by b2 aqueous percentage variable has predominant effect on retention time. The variable flow rate affect considerable on retention time. The flow rate has negative response over Rt whereas % aqueous has positive effect on Rt. From the overall analysis table the relationship between the variables and response was Non-linear. But variable aqueous and flow rate have considerable effect on Retention time. The following equation were obtained for retention time Y1=11.0388+0.618761X1+6.7338 X2+0.5738 X1X2 -2.2662X3-0.1462 X1X3-1.3163X2X3-0.1263X1X2X3……Eq No 2. For the above model the coefficient of determination (r*2*) for the above was 0.9555. Hence the Process model is well valid to predict the behavior of the process and can be used for simulation of the process model.

Design space

Method Operable Design Region (MODR) used for establishment of a multidimensional space based on method factors and settings; MODR can provide suitable method performance. It is also used to establish meaningful method controls such as system suitability, Retention time, and Resolution. There are different combination which solves the problem of method failure like keeping X3 constant and varies X1, X2 or X2 constant and varying X1, X3 or X1 constant and varying X2X3. These combinations and their coefficient values on Y1 (retention time), Y2, Y3, Y4 (Resolution between impurities and Drug) were listed in table no 7. These combinations were shown in contour space which is called as design space.

Retention Time of Drug

Y1: the method response Y1 is retention time of drug, the target which fixed was not to be more than 8min to achieve specificity as per ICH where as Y2, Y3, Y4 is resolution between impurities and drug , USP required only 2 so target was set more than 3. Specificity is the major important factor first and prior considered in Stability indicating assay to avoid method failure. In contour 1 the green zone shows the retention time more than 8 and till 12 min. the green zone provides largest space that ca accompanies the possible robust design and other Rt requirements were 3 to 6 red zone, 6 to 8 min white zone relatively less. It is also noted that the curvature effect was significant I above -1 (X1). The mobile phase less than -1 level likely to elute a drug at void less than 3 min. However, the effect of pH was found to be negligible if the mobile phase is above zero level and the pH is -1 to +2 levels at the flow rate of 1.1 mL/min. In contour 2&3 the green space was found to be relatively less indicates that the higher flow rate always preferred i.e. 0 to +2 levels. It was supported by contour 4 showed a considerable space similar to that of contour 1 at 1.2 mL/min.

Resolution between Impurities and Active Drug

Y2: In contour 5,6,7,8 the yellow region was only the region which is not suitable for the resolution of more than 3. The red region suitable of resolution of 3-6 & white region are beyond resolution 6. So these contour shows that except the two extreme level of pH i.e. below -1(3.5) & above +2 (pH5) level at any aqueous percentage always acceptable. Still based on the contour 5 which indicates that %aqueous at zero level may likely to affect resolution at pH above +1 level & below -1 level. Hence the method is recommended to choose the aqueous percentage more than +1 level where the pH not interferes in the resolution.

Y3: In contour 9, 10, 11, 12 which supported the same where the design space of aqueous phase more than zero level &pH id more than zero level was preferred. All contour showed that irrespective of flow rate the possible design towards + level of aqueous phase is preferred.

Y4: In contour 13, 14, 15, 16indicates that variable pH and aqueous phase percentage + levels were more preferred to acquire adequate resolution. Based on the above analysis the MODR for proposed method was given in table no 8. The overall analysis of method operable design region it suggest that at the flow rate 1.1 mL/min, the higher levels of pH and % aqueous phase will provide suitable design space.

Method verification and its optimized method conditions:

From the design space, for robust process's optimum method conditions to reach desired goals include retention time and resolution were obtained which contains the flow rate of 1.1 mL/min with mobile phase composition of Methanol and %aqueous phase ratio was 60:40 at pH 5 which was verified. The chromatogram was shown in fig 6.

Method Validation

As per ICH Q2 guidelines the method has been validated for its parameters like Linearity, precision, robustness, Accuracy, LOD and LOQ.

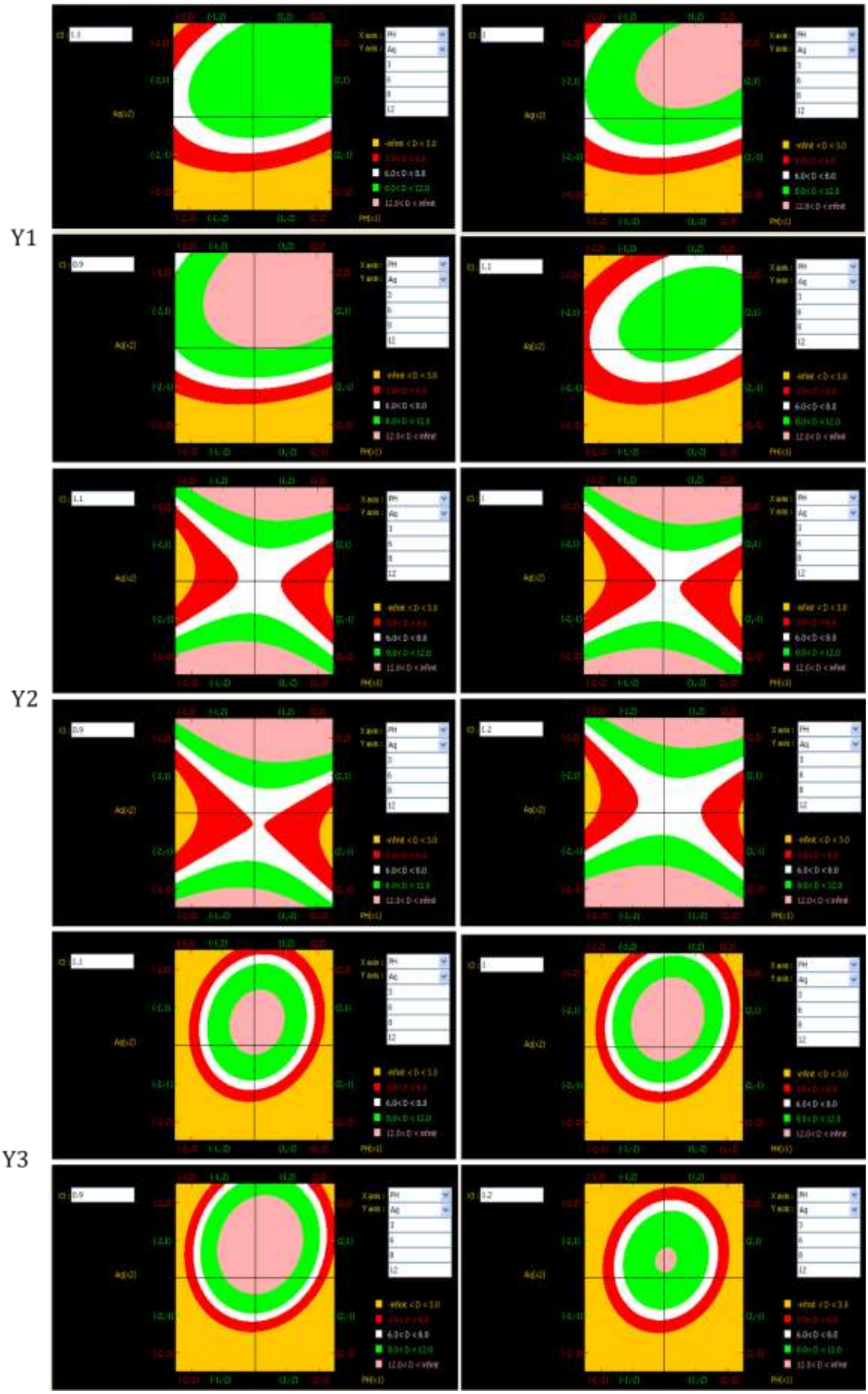

Figure 5: Contour Space

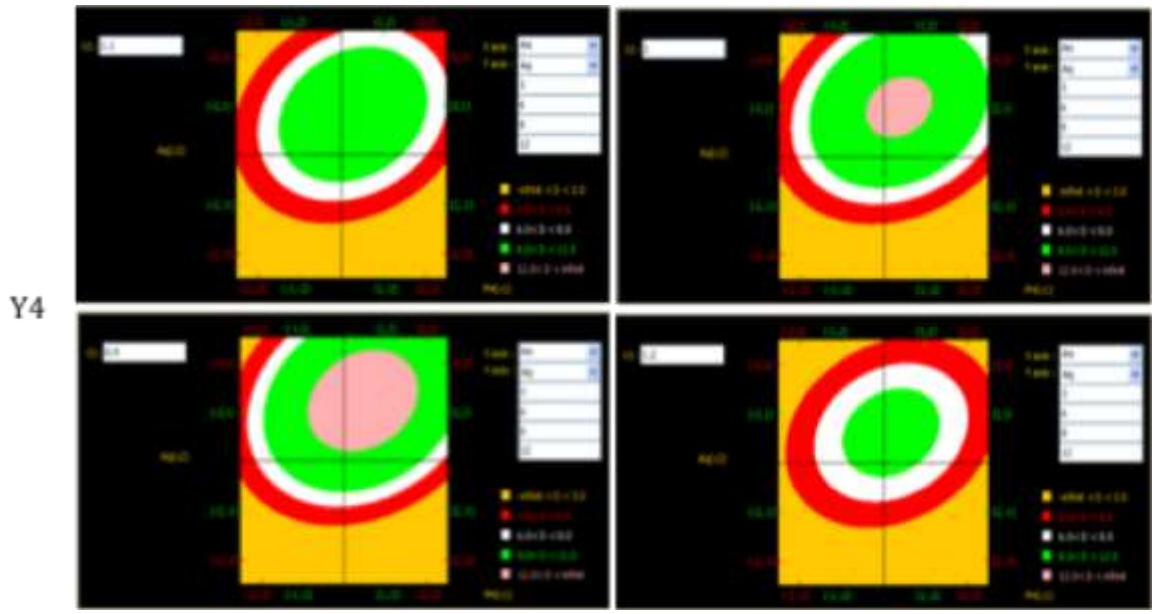

Figure 6: Contour Space

Table 8: Different method operable design region for all contour design at 1.1 ml/ min flow rate

Method response	Variable coded value		Variable absolute value	
	X1	X2	pH (X1)	% aqueous(X2)
Y1	-1 to +2	0 to +2	3.5 – 5	35-45
Y2	0 to +2	+1 to +2	4 – 5	40-45
Y3	-1 to +2	+1 to +2	3.5 -5	30-45
Y4	-2 to +2	-1 to +2	3 -5	25-45

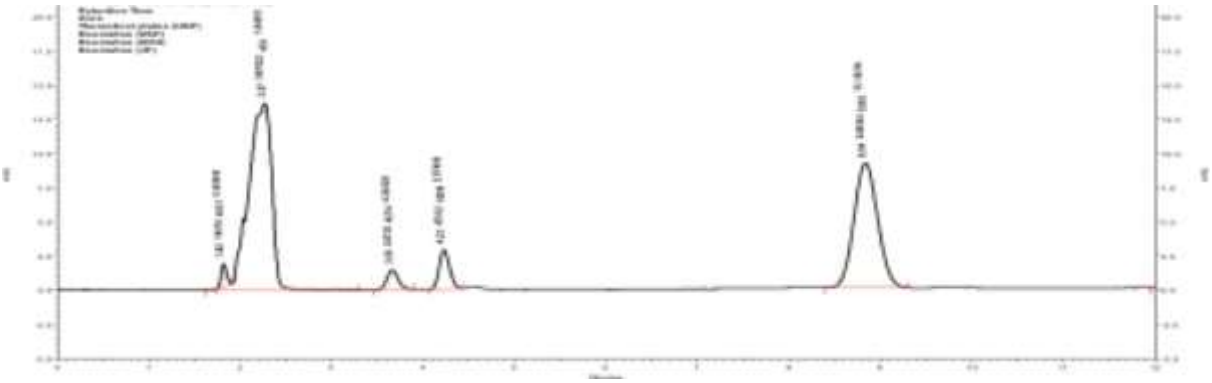

Figure 7: Optimized Chromatogram of Alkali Degradation of Sofosbuvir

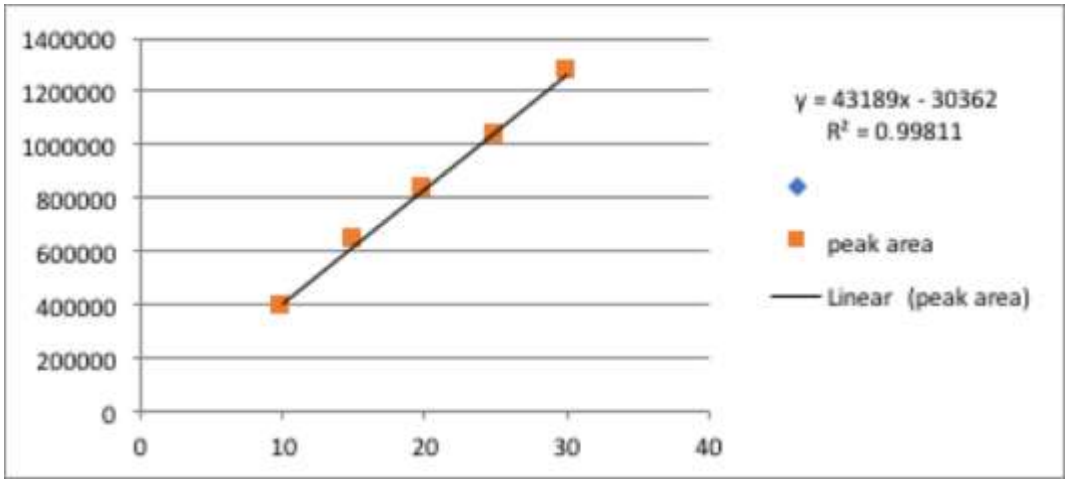

Figure 8: Calibration curve of Sofosbuvir

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Linearity

The linearity of Sofosbuvir was studied in the range of 10 to 30 µg /ml, calibration curve was plotted by peak area responses versus concentrations. It shows linear response (Figure 7) and the regression equation was Y= 43189x-30362(R^2=0.998).

Repeatability

The repeatability of the method was checked over six replication of 10 µg/ml, it shows %RSD was 0.30188%.

Robustness

Robustness of the method was proved by changing the flow rate, pH of aqueous phase and Mobile phase compositions with in MODR region. The flow rate was tested from 1.1 ml/min -1.3 ml/min. the % RSD was 1.3 % and the % of aqueous phase was tested up to 4% it shows % RSD less than 2 which indicates the proposed method was robust.

CONCLUSION

There is a need to implement QbD approach into the analytical method development and validation to encounter the method failure. An alarming rate of awareness on the importance of implementing the QbD approach has increased. So, an attempt was made and developed a simple, robust, economic QbD based stability indicating RP-HPLC method for estimation Sofosbuvir in bulk drug. Instead of normal trial and error method, the QbD approach enables us to understand the critical analytical attributes about the drug by constructing the Analytical target profile. Normal method was developed for estimation of Sofosbuvir using Methanol: water 65:35 %v/v, at flow rate of 1 mL/min at 261 nm. Forced degradation was conducted under various stress conditions like alkali hydrolysis, acid hydrolysis, oxidative degradation, photolytic degradation & thermal degradation. Among these the alkali degradation was shows relatively more number of impurities and also two critical impurities likely to co-elute with the active drug. Hence, the alkali degraded sample has been taken into consideration for method optimization under Quality by Design approach. Based on the risk assessment the critical method parameters were identified like pH of aqueous phase, % of aqueous phase and flow rate and there effect on method response like retention time and resolution were studied. The CCD method was employed to know the interaction effect among the variables. The experimental model shown coefficient value was0.9555. The method operable design region was proposed form contour and the optimized chromatographic conditions were selected and method was verified. The proposed method was validated as per ICH guidelines.

The QbD approach has given a method for estimation of Sofosbuvir and its degradants below 9 min. a well separated impurities and drug substance with resolution more than 2 which indicates the specificity of the method which further can utilize the quantification of impurities with out interference. The proposed method was not utilizing any buffer it was developed by using methanol and water which is cost effective and simple. The MODR region gives design space to establish the robustness of method. Hence the developed QbD approach stability indicating method for the estimation of Sofosbuvir was simple, cost effective, selective& accurate.

ACKNOWLEDGEMENT

The authors gratefully thank to Research laboratory of Raghavendra Institute of Pharmaceutical Education and Research, Andhra Pradesh, India for utilizing the QbD software and also thank to Dr. Ramalingam Peraman for his valuable support and advice on QbD approach.

Conflict of interest: The authors declare that they have no conflict of interest.

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