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IMPLEMENTATION OF QUALITY BY DESIGN APPROACH TO DEVELOP AND VALIDATE STABILITY INDICATING ASSAY METHOD FOR SIMULTANEOUS ESTIMATION OF SOFOSBUVIR AND LEDIPASVIR IN BULK DRUGS AND TABLET FORMULATION

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Keywords:

Sofosbuvir, Ledipasvir, Stability indicating an assay method, Validation, QbD

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ABSTRACT: This study developed a stability-indicating reversed-phase high-performance liquid chromatography method for simultaneous estimation of sofosbuvir and ledipasvir of bulk drug and formulation by using a QbD approach. Literature survey reveals that there were very few analytical methods available for the simultaneous estimation of sofosbuvir and ledipasvir. An attempt was made to develop and validate stability indicating assay method using RP-HPLC through QbD approach. In a QbD approach, Box-Behnken screening based on critical method parameters, *i.e.* (Buffer pH, organic phase-% methanol and flow rate). The interaction effect of these parameters on the response variables (Retention Time, NTP and tailing factor) were evaluated through 3D response graphs. The plots revealed the final chromatographic conditions of the method. The chromatographic separation was achieved using C18 inertsil ODS-2, 250 mm × 4.6 mm × 5µ column, buffer ammonium acetate and PDA detector at 254 nm. The developed method was successfully applied for the determination of the two drugs from its pharmaceutical formulation as well as successfully applied for forced degradation studies. Force degradation studies include acid hydrolysis, base hydrolysis, oxidation, thermal degradation and photolytic degradation of sofosbuvir and ledipasvir in combination. The methods can be used for routine analysis of formulations containing any of the above drugs or combinations without any alteration in the chromatographic conditions.

INTRODUCTION: The fundamental premise behind QbD is that quality is 'designed' into the process at the onset to establish a thorough understanding of the response of the system quality to system parameters, leading ultimately to the establishment of the design space for the method. The QbD approach is a recent trend in analytical method development, and it helps a lot if get properly implemented.

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QbD approach suggests looking into the quality of the analytical process during the development stage itself. It says that quality should be built into the process design rather than testing into final results of analytical process [1]. Traditional chromatographic method development has always involved the time-consuming process of varying one system parameter at a time, examining its effect on the method and system operation.

This generally requires a large number of experimental runs, and in most situations, the developed method requires further development. Hepatitis C virus (HCV) infection is a significant public health concern, with approximately 150-170 million infected individuals worldwide. The present assessment finds a global prevalence of 2.35%,

affecting 160 million chronically infected individuals [2]. Patients infected with HCV are at risk of life-threatening complications and can lead to cirrhosis, decompensated liver disease (liver failure), hepatocellular carcinoma, and the need for liver transplantation [3]. Combination of two direct-acting antiviral drugs that target different steps of the hepatitis C virus (HCV) lifecycle. Sofosbuvir is a nucleotide analogue HCV polymerase inhibitor; it blocks the polymerase enzyme which the virus must use to reproduce. Ledipasvir is an HCV NS5A replication complex inhibitor that interferes with another protein HCV uses to reproduce [4].

Very few stability indicating assay methods are reported for the simultaneous determination of sofosbuvir and ledipasvir in the presence of their degradants and their associated main impurities. Otherwise, there are few HPLC [5, 6], LC-MS [7, 8] procedures known for the analysis of sofosbuvir and ledipasvir individually. The procedure gave acceptable results with fresh products but gave overestimation during analysis of stability samples and aged products. Therefore, the focus in the present study was to develop a stability-indicating HPLC method for the combination, by degrading the drugs together under various stress conditions according to ICH. The drugs were separated from degradation products on a reversed-phase HPLC column.

MATERIAL AND METHODS:

Chemicals and Reagents: Pure API, as well as combination products of sofosbuvir and ledipasvir, were obtained as gift samples from Mylan Pharmaceuticals Private Limited, Hyderabad, India. HPLC grade methanol and water were purchased from Merck, Mumbai. Buffer materials and all other chemicals were of analytical- reagent grade.

Equipment: The HPLC system consisted of 'Jasco PU-2089 Plus Quaternary Gradient' pump and 'Jasco MD-2018 Plus' photodiode array detector. The chromatographic separations were performed using C18 Inertsil ODS (250 mm × 4.6 mm × 5µ) column, eluted with mobile phase at the flow rate of 1.0 ml/min. The mobile phase consisted of methanol and 0.01M ammonium acetate buffer (70:30 v/v), apparent pH adjusted to 3.5 with 1.0 N Glacial acetic acid solution, filtered through a 0.45m nylon filter and degassed in an ultrasonic bath before use. Measurements were made with an injection volume of 20 µl and ultraviolet (UV) detection at 254 nm. For the analysis of forced degradation samples, the photodiode array detector was used in scan mode with a scan range of 220-400 nm and desired peak coverage of 100%. The output signal was integrated using chromnav software. Peak homogeneity was expressed regarding peak purity values and was obtained directly from the spectral analysis report obtained using the software as mentioned above.

Solution Preparation:

Preparation of Standard Mixture Solution: For RP-HPLC, a standard stock solution was prepared in methanol containing 1000 µg/mL of sofosbuvir and 225 µg/mL of ledipasvir. Pipette out 1 mL from standard stock solution into a 10 mL volumetric flask and makeup with methanol to get the working standard solution containing 100 µg/mL of sofosbuvir and 22.5 µg/mL of ledipasvir.

Preparation of Sample Solution: Ten tablets were opened and transferred the contents (each tablet containing 400 mg of sofosbuvir and 90 mg of ledipasvir) equivalent to 100 mg of Sofosbuvir and 22.5 mg of ledipasvir into a 100 mL volumetric flask. The 60 mL of mobile phase was added into the volumetric flask. The final volume was made up to 100 mL and prepared a stock solution containing 1000 µg/mL of sofosbuvir and 225 µg/mL of ledipasvir. Aliquots of the stock solutions were appropriately diluted with mobile phase to obtain a working sample solution containing 100 µg/mL of sofosbuvir and 22.5 µg/mL of ledipasvir.

Initial Method Development:

Choice of Wavelength: Optimum wavelength of 254 nm was selected based on sufficient response at isobestic point of sofosbuvir and ledipasvir and less baseline noise during the chromatographic run.

Choice of Diluent: A suitable diluent for sofosbuvir and ledipasvir was selected by checking its solubility in methanol, acetonitrile, and distilled water. Finally, methanol was chosen based on the solvent which gave optimum solubility.

Choice of Column: different stationary phases, C8, and C18 columns along with mobile phase constituted of different ratio of water/buffer, acetonitrile and methanol were tried to get the

desired separation of peaks. After carrying out the basic trials, C18 column shows optimum retention than C8 column. Hence, C18 Column was selected for further method development.

Method Optimization by QbD Approach:

QbD (Quality by Design) Approach for Simultaneous RP-HPLC Method Development: The concepts described in ICH guidelines Q8 to Q10 are commonly referred to as QbD in a nutshell. QbD can be defined as a systematic approach which begins with a predefined objective, and it mainly focuses on the product, its process and its control based on logical and profound knowledge of the science involved and quality risk management [9]. When this concept is applied to analytical methods, it is termed 'Analytical QbD.' Sofosbuvir is more polar than ledipasvir. Ledipasvir has more pKa value than sofosbuvir hence ledipasvir is insoluble in water, and it gets precipitate in water. Different combinations of water and methanol were used for optimization of mobile phase as shown in the table. Initial trials data concluded that optimization of mobile phase for sofosbuvir and ledipasvir is quite difficult because of the compatibility of two substances with solvents is different from each other. Hence, an attempt has been made to apply QbD approach to develop simultaneous method development of sofosbuvir and ledipasvir.

Following Steps can divide method Development using Quality by Design Approach:

Analytical Target Profile: Primary aim was to develop a more robust method and validation of a developed method for simultaneous estimation of sofosbuvir and ledipasvir. Quality by Design approach was applied to get MODR (Method Operable Design Region). ATP is the target setting process in the approach of developing the analytical method. The method performance expectations such as specificity, linearity, range, precision, accuracy, detection limit, quantification limit, robustness, and system suitability assumed as predefined targets according to the ICH guidelines for the analytical method development.

Risk Assessment (RA): QRM (ICH Q9) is 'a systematic process for the assessment, control, communications and review of risks to the quality across the lifecycle. RA is an integral part of the analytical QbD process. Their use facilitates identification and ranking of parameters that could impact method performance and conformance to the ATP. Risk assessment approaches which begin with mapping tools such as flow-chart and Ishikawa fishbone diagram as shown in **Fig. 1** to evaluate risk.

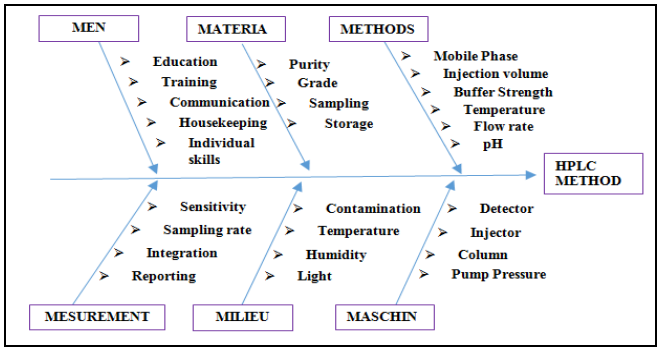

FIG. 1: ISHIKAWA FISHBONE DIAGRAM

Design Space: The multinational combination and interaction of input variables and process parameters that have been demonstrated to assure the quality of the data produced by the method. Selection of CPP & CQA is an integral part of Design space formation.

Critical Quality Attribute (CQA): CQA is a "physical, chemical, biological property or characteristic that should be within an appropriate limit, range to ensure the desired product quality." Tailing factor, NTP and retention time achievement considered as critical quality attributes for this method as shown in **Table 1**.

Critical Process Parameters (CPP): Critical method parameters, *i.e.* flow rate, buffer pH and organic modifier are process inputs that have a direct and significant influence on CQAs when they are varied within regular operation range. The range of the CPP was selected from the data obtained in initial trials.

Control Strategy: From the aspect of AMD view, the control strategy may be defined as 'the controls on input factors to a method that ensure the method meets both traditional criteria and wider performance related goals.' The controls can include the quality of drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls and frequency of monitoring and control (ICH Q10).

Continuous Improvement throughout Product Life Cycle: Method performance can be improved throughout the method development. The analyst has opportunities to placed inventive approaches to improve quality. Process performance can be monitored to make sure consistency in quality. The QbD approach avails the continuous improvement throughout the method life cycle; this is the distinguishing point from the conventional method which is a much-frozen process.

RESULTS AND DISCUSSION:

Software Aided Method Optimization: The Box-Behnken design is an independent quadratic design in that it does not contain an embedded factorial or fractional factorial design. In this design, the treatment combinations are at the midpoints of edges of the process space and the center. These designs are rotatable (or near rotatable) and require 3 levels of each factor. The designs have limited capability for orthogonal blocking compared to the central composite designs.

Statistical analysis was used to identify the significant influential chromatographic factors and their interaction impact on the six responses, *i.e.* retention time, tailing factor, NTP of both sofosbuvir and ledipasvir. The analysis of 3D response surface plots and predicted *vs*. actual plots were used to estimate as to which method parameter gave the most acceptable responses as shown in **Fig. 2-13**. The response variables, *i.e.* retention time, tailing factor and NTP was statistically evaluated as given in **Table 1**.

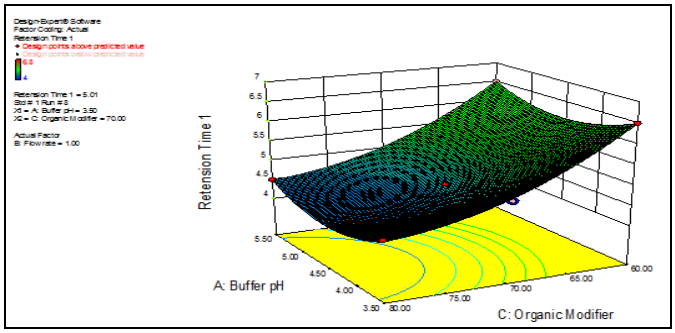

FIG. 2: EFFECT OF pH AND ORGANIC MODIFIER ON RETENTION TIME OF SOFOSBUVIR

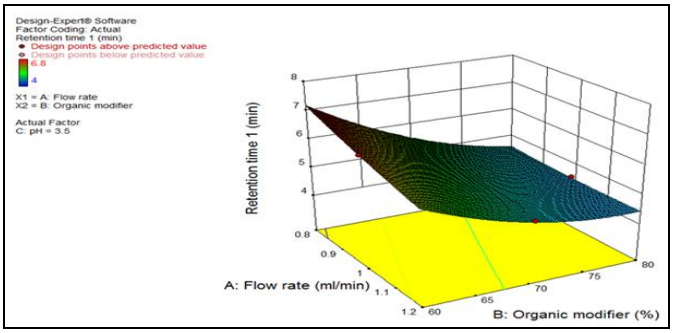

FIG. 3: EFFECT OF FLOW RATE AND ORGANIC MODIFIER ON RETENTION TIME OF SOFOSBUVIR

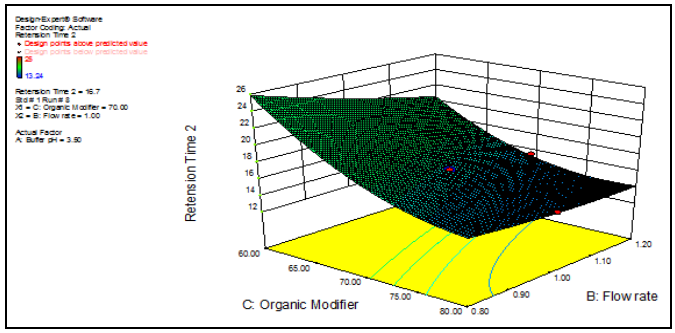

FIG. 4: EFFECT OF ORGANIC MODIFIER AND FLOW RATE ON RETENTION TIME OF LEDIPASVIR

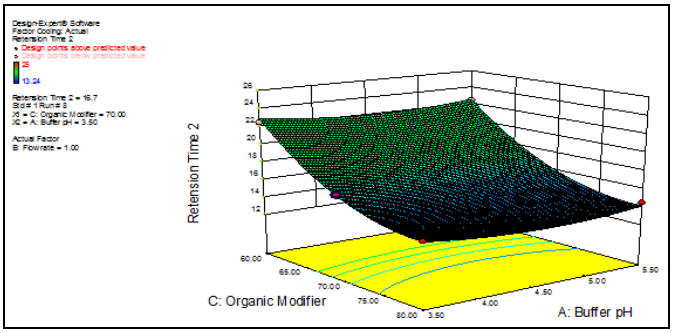

FIG. 5: EFFECT OF ORGANIC MODIFIER AND BUFFER pH ON RETENTION TIME OF LEDIPASVIR

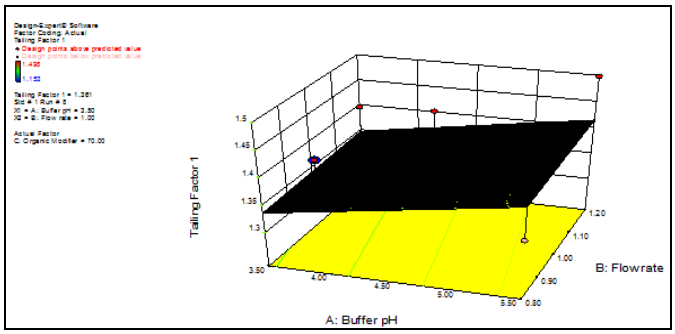

FIG. 6: EFFECT OF BUFFER pH AND FLOW RATE ON RETENTION TIME OF SOFOSBUVIR

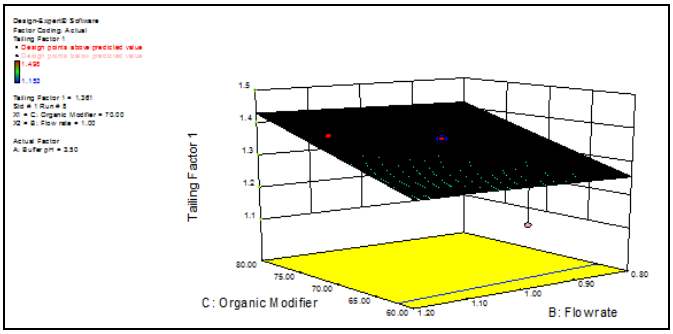

FIG. 7: EFFECT OF ORGANIC MODIFIER AND FLOW RATE ON RETENTION TIME OF SOFOSBUVIR

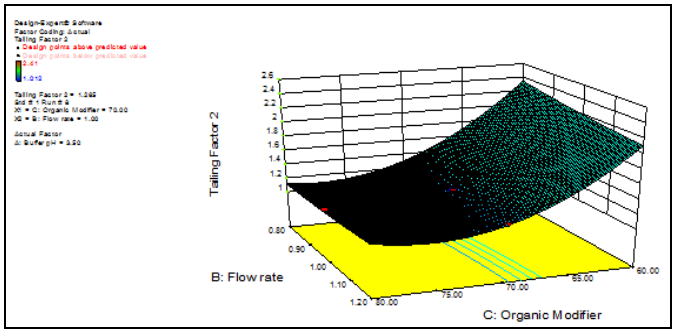

FIG. 8: EFFECT OF ORGANIC MODIFIER AND FLOW RATE ON TAILING FACTOR OF LEDIPASVIR

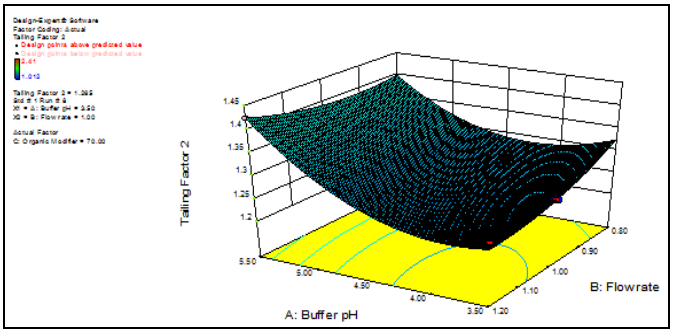

FIG. 9: EFFECT OF BUFFER pH AND FLOW RATE ON TAILING FACTOR OF LEDIPASVIR

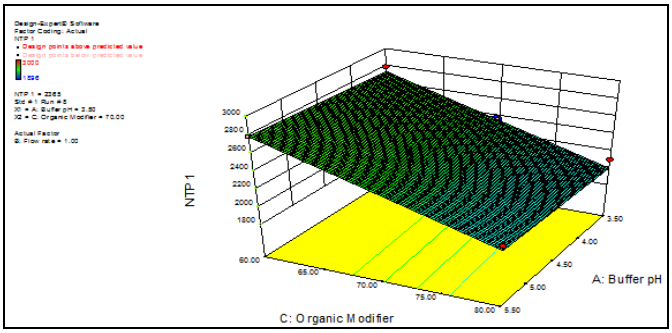

FIG. 10: EFFECT OF ORGANIC MODIFIER AND BUFFER pH ON NTP OF SOSOFOSBUVIR

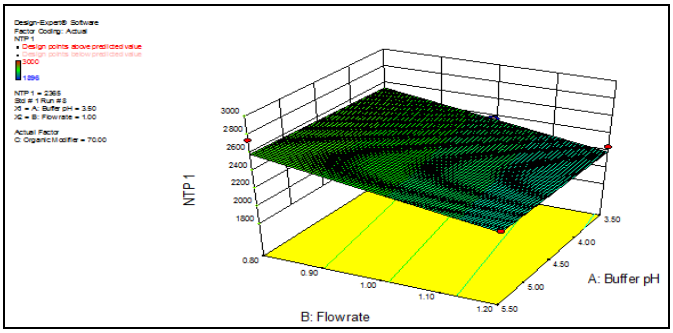

FIG. 11: EFFECT OF FLOW RATE AND BUFFER pH ON NTP OF SOFOSBUVIR

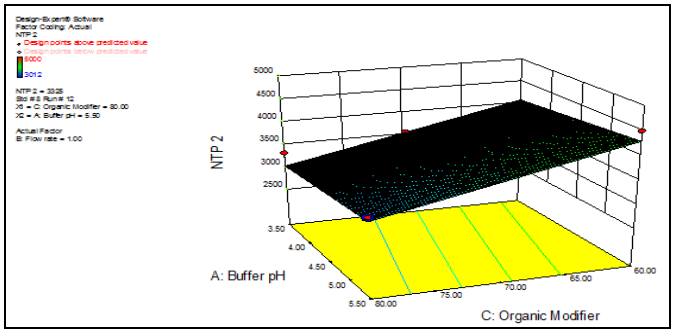

FIG. 12: EFFECT OF ORGANIC MODIFIER AND BUFFER pH ON NTP OF LEDIPASVIR

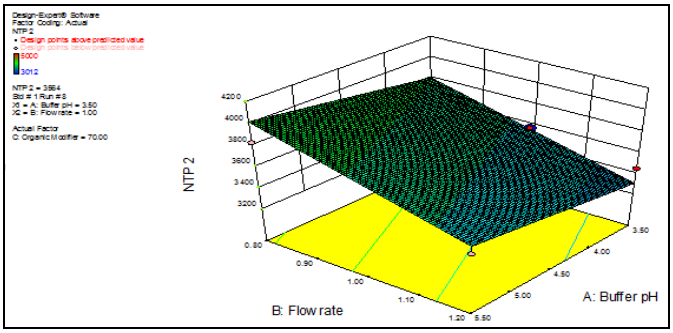

FIG. 13: EFFECT OF FLOW RATE AND BUFFER pH ON NTP OF LEDIPASVIR

TABLE 1: FACTORS SCREENING BY BOX- BEHNKEN DESIGN

Run	(Factor) Flow rate ml/min	(Factor) Organic modifier %	Factor pH	Response Rt I	Response tailing factor I	Response NTP I	Response Rt II	Response tailing factor II	Response NTP II
1	1.2	80	4.5	4.00	1.428	1896	13.24	1.128	3028
2	1	70	4.5	4.70	1.476	2198	15.58	1.259	3521
3	1	80	5.5	4.50	1.454	2100	14.50	1.268	3325
4	0.8	60	4.5	6.80	1.398	3000	25.00	2.410	5000
5	1.2	70	5.5	4.80	1.498	2252	15.50	1.424	3450
6	1.2	60	4.5	5.50	1.275	2465	20.12	2.210	3725
7	1	60	3.5	6.50	1.153	2789	22.50	2.150	3654
8	1	70	3.5	5.01	1.361	2365	16.70	1.265	3564
9	0.8	80	4.5	4.20	1.474	1996	14.00	1.012	3012
10	1	80	3.5	4.50	1.385	2100	14.50	1.183	3325
11	1	60	5.5	6.50	1.265	2786	22.45	2.350	4500
12	1.2	70	3.5	4.80	1.398	2252	15.50	1.257	3450
13	0.8	70	5.5	5.50	1.345	2745	17.30	1.384	3833

Validation:

System Suitability: System suitability test was carried out to verify that the analytical system is working properly to give accurate and precise results. Standard solution (10 µg/ml sofosbuvir and 2.25 µg/ml ledipasvir) was injected six times, and the chromatograms were recorded. The % RSD for area response obtained from six replicate injections

of Standard solution should be 2.0 %, tailing factor should be 2.0. Theoretical plates should be 2000 and resolution of drug peaks should be 2.0.

Linearity: The linearity peak area response was determined by analyzing solutions having concentrations in the range of 50-150 µg/ml and 11-33 µg/ml for sofosbuvir and ledipasvir respectively from the same solution. Peak area of each solution was measured using the developed method. Calibration curve of peak area *vs.* concentration was plotted. The correlation coefficient and regression line equations for sofosbuvir and ledipasvir were determined.

Precision: 6 replicates of standard mixture solution having and sofosbuvir (50, 100 and 150 µg/ml) and ledipasvir (11, 22, 33 µg/ml) were prepared, and chromatograms were recorded and RSD was calculated.

Intraday Precision: Standard solutions containing sofosbuvir (50, 100 and 150 µg/ml) and ledipasvir (11, 22 and 33 µg/ml) were analyzed 3 times on the same day. Chromatogram of each sample was recorded. SD and RSD were calculated.

Interday Precision: Standard solutions containing sofosbuvir (50, 100 and 150 µg/ml) and ledipasvir (11, 22 and 33 µg/ml) were analyzed on three different days. Chromatogram of each sample was recorded.SD and RSD were calculated.

Accuracy: % Recovery studies were carried out by spiking known amount of sofosbuvir and ledipasvir in the concentration of 50%, 100% and 150% of the working level in triplicates. Chromatogram of each spiked solutions was taken, and the total amount of drug was calculated and from which % recovery was calculated.

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD and LOQ were obtained by successively decreasing the concentration of sofosbuvir and ledipasvir as long as a signal to noise ratio of not less than 3:1 and 10:1 is maintained respectively. The representative chromatograms for LOD and LOQ were recorded.

Solution Stability: The stability studies were evaluated for three different concentrations *i.e.* 50 µg/mL, 100 µg/mL and 150 µg/mL for sofosbuvir and 11 µg/mL, 22 µg/mL and 33 µg/mL standard solutions for ledipasvir. Six replicates of each which were stored refrigerated temperature (2-8°C) for a week. The sample analysis was performed at initial time zero and after those 6, 12, 24, 48 and 72 h.

TABLE 2: STATISTICAL DATA OF VALIDATION

Parameters	Sofosbuvir	Ledipasvir
Linearity range	50-150 µg	11-33µg
Regression equation	Y=24184x – 61274	Y=6289.5x + 27580
Correlation coefficient	R^2=0.9997	R^2=0.9998
% Recovery (n =3)	100.206 %	99.983 %
LOD	0.45 µg/ml	3.85 µg/ml
LOQ	1.5 µg/ml	11 µg/ml
Precision (% R.S.D.)		
Intra-day (n = 3)	0.0025 %	0.0039 %
Inter-day (n= 3)	0.0032 %	0.2447 %
Specificity	no other interfering peak around the retention time of Sofosbuvir	no other interfering peak around the retention time of Ledipasvir
Solution Stability (% RSD)	0.0155%	0.0184%

Assay of Tablet Formulation: Sample solution containing 100 µg/ml of sofosbuvir and 22.5 µg/ml was injected into the chromatographic system, and peak area was measured for sofosbuvir and ledipasvir. The percentage of label claim of sofosbuvir and ledipasvir were calculated and was found to be 99.80% and 99.55% respectively. A representative chromatogram is as shown in **Fig. 14**.

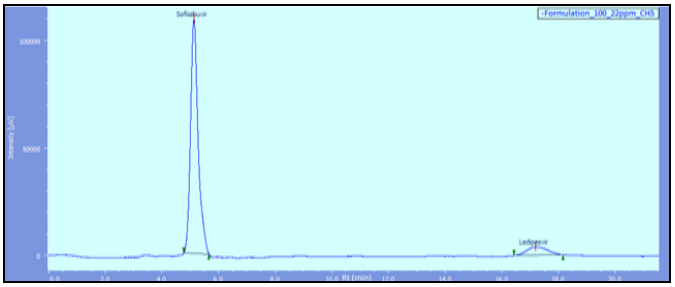

FIG. 14: REPRESENTATIVE CHROMATOGRAM

Force Degradation: To prove the stability indicating nature of the method, forced degradation studies were carried out by exposing the stock solution of the drug to the following conditions:

1. Acid hydrolysis:
2. Base hydrolysis:
3. Oxidation degradation:
4. Thermal degradation:
5. Photolytic degradation:

Acid Hydrolysis: For acid hydrolysis**,** 50 mg of sofosbuvir and 11 mg ledipasvir was accurately weighed and transferred to a round bottom flask containing 5 ml of diluent. Then**,** 5 ml of 1N HCl was added in a round bottom flask, and this mixture was refluxed on a water bath for 1 h at 80 °C. After the reflux, the round bottom flask containing the stressed solution was cooled to room temperature, transferred to 50 ml volumetric flask, neutralized with the corresponding base and volume was made up with diluent (Solution A). Take 1 ml of solution A and transferred into 10 ml of volumetric flask, make up the volume with diluent. Finally, this solution was loaded into HPLC, and the corresponding Chromatogram was recorded as given in **Fig. 15**.

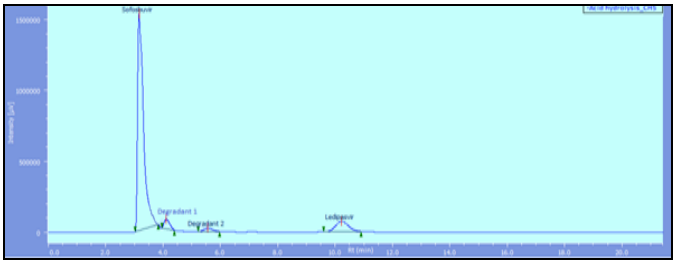

FIG. 15: CHROMATOGRAM WAS RECORDED

Base Hydrolysis: For acid hydrolysis**,** 50 mg of sofosbuvir and 11 mg ledipasvir was accurately weighed and transferred to a round bottom flask containing 5 ml of diluent. Then, 5 ml of 1N NaOH was added in a round bottom flask, and this mixture was refluxed on the water bath for 1 h at 80 °C. After the reflux, the round bottom flask containing the stressed solution was cooled to room temperature, transferred to 50 ml volumetric flask, neutralized with the corresponding acid and volume was made up with diluent (Solution A). Take 1ml of solution A and transferred into 10 ml of volumetric flask, make up the volume with diluent. Finally, this solution was loaded into HPLC, and the corresponding chromatogram was recorded as given in **Fig. 16**.

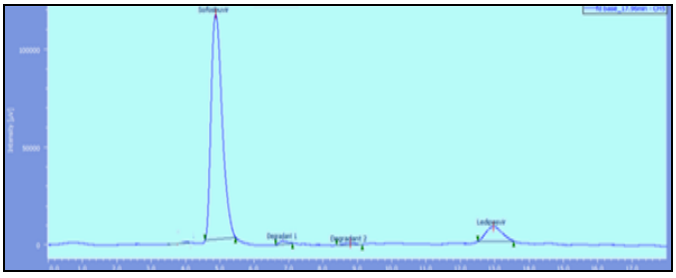

FIG. 16: CORRESPONDING CHROMATOGRAM WAS RECORDED

Oxidative Degradation: For oxidative degradation, 50 mg of sofosbuvir and 11 mg ledipasvir was accurately weighed and transferred 50 ml volumetric flask containing 5 ml of diluent. Then, 5 ml of hydrogen peroxide (3%) was added to this flask, and the mixture was kept as such at room temperature for 30 min. After 30 min, the volume was made up with diluent (Solution A). Take 1 ml of solution A and transferred into 10 ml of volumetric flask, make up the volume with diluent. Finally, this solution was loaded into HPLC, and the corresponding chromatogram was recorded as given in **Fig. 17**.

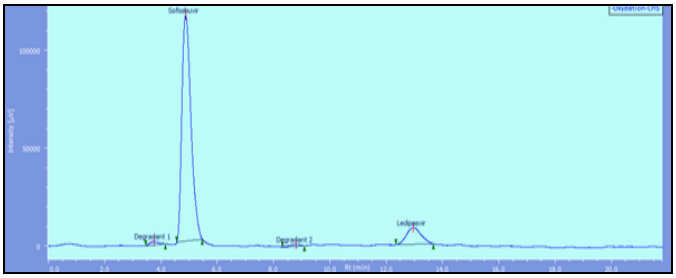

FIG. 17: CORRESPONDING CHROMATOGRAM WAS RECORDED

Thermal Degradation: For thermal degradation study, 50 mg of sofosbuvir and 11 mg ledipasvir was accurately weighed and transferred to a round bottom flask containing 10 ml of diluent. Then, this solution was refluxed on a water bath for 2 h at 80°C. After the reflux, the round bottom flask containing the stressed solution was cooled to room temperature, transferred to 50 ml volumetric flask and volume were made up with diluent (Solution A). Take 1 ml of solution A and transferred into 10 ml of volumetric flask, make up the volume with diluent. Finally, this solution was loaded into HPLC, and the corresponding chromatogram was recorded as given in **Fig. 18**.

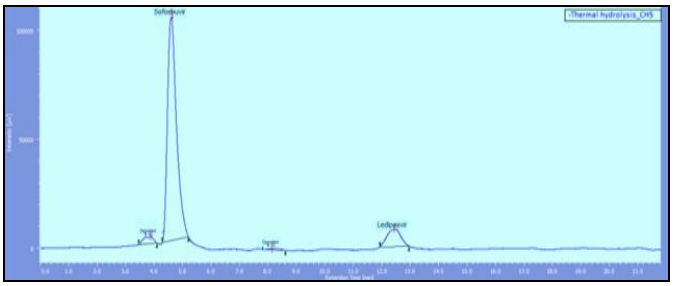

FIG. 18: CORRESPONDING CHROMATOGRAM WAS RECORDED

Photolytic Degradation: The photolytic degradation was carried out by exposing drug substance, *i.e.* sofosbuvir (50 mg) and ledipasvir

(11 mg) under UV light at 290 nm for one week. After 1 week, the drug substance was transferred in 50 ml volumetric flask add about 10 ml of methanol (diluent), sonicated to dissolve and make up with diluent (Solution A). Take 1 ml of solution A and transferred into 10 ml of volumetric flask, make up the volume with diluent. Finally, this solution was loaded into HPLC, and the corresponding chromatogram was recorded as given in **Fig. 19**.

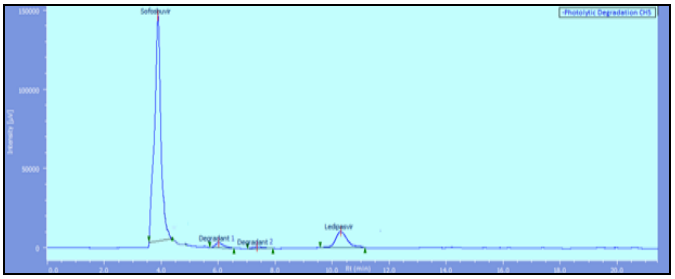

FIG. 19: CORRESPONDING CHROMATOGRAM WAS RECORDED

TABLE 3: RESULT OF FORCED DEGRADATION STUDIES

S. no.	Degradation condition	% Degradation of SFS	Rt of degradation products (min)	% Degradation of LPV	Rt of degradation products (min)
1	Acid hydrolysis	3.10	4.116	17.13	5.591
2	Base hydrolysis	7.80	5.861	12.45	9.251
3	Oxidation	1.40	3.710	11.91	8.741
4	Thermal degradation	3.20	3.914	9.20	8.295
5	Photolytic degradation	0.00	No degradation	18.72	6.001,7.371

DISCUSSION: Forced degradation studies done on sofosbuvir and ledipasvir indicate that sofosbuvir was degraded by 3.1%, 7.8%, 1.40% and 3.2% when subjected to acid hydrolysis, base hydrolysis, oxidation degradation and thermal degradation respectively and ledipasvir was degraded by 17.13%, 12.45%, 11.91%, 9.20% and 18.72 when subjected to acid hydrolysis, base hydrolysis, oxidation degradation, thermal degradation, and photolytic degradation respectively. The representative chromatograms of the forced degradation studies reveal that all the degradation products were fully resolved, this indicates the specificity of the method. Thus the method can be employed for monitoring the stability of sofosbuvir and ledipasvir in bulk drug.

These studies also determine the physical and chemical stability of drug substance and drug product which may be further useful to storage conditions for the drug product. Since, sofosbuvir and ledipasvir are susceptible to oxidation at room temperature, sofosbuvir and ledipasvir tablets should be stored in a dry place as moisture is a catalyst of oxidation and low-moisture environment may sometimes resolve the problem of oxidation.

Another alternative is to use an oxygen scavenger that helps to control the oxygen level within the headspace of a drug's primary packaging. This may help to maintain the drug potency and other properties under extended and variable storage and shelf conditions.

CONCLUSION: The simultaneous RP-HPLC assay method developed by QbD approach for determination of sofosbuvir and ledipasvir is linear, accurate, precise, rapid and specific as evident from the validation results. The developed method is also stability indicating and can be conveniently used for quality control to determine the assay in regular sofosbuvir and ledipasvir product development, production and stability samples.

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CONFLICT OF INTEREST: Nil

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