Research Article

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Stability indicating method development and validation for the simultaneous estimation of ledipasvir and sofosbuvir in bulk drug by using RP-HPLC

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ARTICLE INFO

ABSTRACT

A new, simple, precise, accurate, and reproducible RP-HPLC for stability-indicating method development and validation of Ledipasvir and Sofosbuvir in bulk form. Separation of Ledipasvir and Sofosbuvir was successfully achieved Dona Zorbax C8, 250 X4.6mm, 5μm or equivalent in an isocratic mode utilizing 0.1% OPA: Methanol (45:55) at a flow rate of 1.0mL/min and the eluate was monitored at 238nm, with a retention time of 3.303 and 7.303minutes for Ledipasvir and Sofosbuvir respectively. The method was validated and their response was found to be linear in the drug concentration range of 45μg/ml to 135μg/ml for Ledipasvirand 200μg/ml to 600μg/ml for and Sofosbuvir. The values of the correlation coefficient were found to 0.999 for Ledipasvirand 1 for Sofosbuvir respectively. The LOQ for Ledipasvirwere found to be 0.695 respectively. The LOQ for Sofosbuvir was found to be 2.2063 respectively. This method was found to be a good percentage recovery for Ledipasvir and Sofosbuvirwere found to be 100 and 100 respectively indicates that the proposed method is highly accurate. The specificity of the method shows a good correlation between retention times of standard with the sample so, the method specifically determines the analyte in the sample without interference from the excipients of tablet dosage forms. The method was extensively validated according to ICH guidelines for Linearity, Accuracy, Precession, Specificity, and Robustness.

Keywords: Ledipasvir; Sofosbuvir; High performance liquid chromatography and UV spectrometry.

INTRODUCTION [1]

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. The current good manufacturing practice (CGMP) and Food Drug Administration (FDA) Guidelines insist for adoption of sound methods of analysis with greater sensitivity and reproducibility [2]. Development of a method of analysis is usually based on prior art (or) existing literature, using the same (or) quite similar instrumentation [3].It is rare today that an HPLC-based method is developed that does not in same way relate (or) compare to existing, literature based approaches. Today HPLC (High performance liquid chromatography) is the method of choice used by the pharmaceutical industry to assay the intact drug and degradation products. In recent years, several analytical techniques have been evolved that combined two or more methods into one called "hyphenated" technique eg: GC/MS, LC/MS etc. The complete Analysis of a substance consists of four main steps.

Newer analytical methods are developed for these drugs or drug combinations of the below reasons [2]

- There may not be suitable method for a particular analyte in the specific matrix.
- Existing method may be too error prone or unreliable (have poor accuracy and precision).
- Existing method may be expensive, time consuming, energy intensive and may not be provide sensitive or analyte selectivity, and not easy for automation.
- Newer instrumentation and techniques may have evolved that provide opportunities for improved methods.
- There may be need for an alternate method to confirm, for legal and scientific reasons.

The newly developed analytical methods having their importance in different fields that include, research and

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development centre (R&D), Quality control department (QC).

AIM OF WORK [2]
To develop a sensitive, simple, rapid, accurate and Stability indicating analytical method for the simultaneous estimation of Ledipasvir and Sofosbuvir and validation

PLAN OF WORK [3]
The plan of proposed work includes the following steps:

a) The extensive survey of literature for drug regarding the physicochemical properties, pharmacological action and analytical methods.

b) Method development by RP-HPLC

- Selection of suitable wave length.
- Selection of chromatographic technique.
- Method development trails
- Optimization of chromatographic condition.

b) Validation of developed method using following parameters as per the ICH guidelines.

- System suitability
- Specificity

Forced degradation studies

- Acidic degradation
- Base degradation
- Oxidative degradation
- Thermal degradation
- Photo degradation
- Linearity
- Precision
- Accuracy
- Robustness
- Limit of quantification

INSTRUMENTS/EQUIPMENTS USED [4]
Instruments:

- WATERS HPLC, Model: Agilent 2695, Photo diode array detector (PDA), with an automated sample injector. The output signal was monitored and integrated using Empower 2 software. ELIPSE C8 (150mm*4.6, 5 mm, Make: Waters) column was used for separations.

List of Equipment's[5]

METHOD DEVELOPMENT TRAIL OPTIMISED:
Observation:RT was found to be good and the peak symmetry of both drugs were good. And the resolution theoretical plate count and tailing were within the limits and it is used for validation of the method.

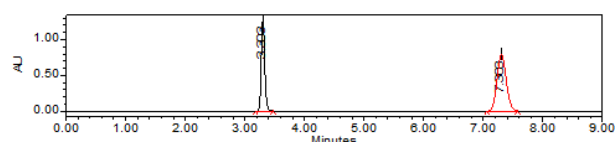| S.NO	Equipment's	Model	Company
1	Electronic Balance	ER200A	ASCOSET
2	Ultra-Sonicator	SE60US	ENERTECH
3	Heating Mantle	BTI	BIO TECHNICS INDIA
4	Thermal oven	———	NARANG
5	pH Meter	AD102U	ADWA
6	Filter Paper 0.45 microns	———	MILLI PORE

Fig 01: Typical chromatogram of optimised conditions

OPTIMIZED METHOD FOR RP-HPLC
Mobile Phase: 0.1% OPA: Methanol (45:55)

Column: Zorbax 250X 4.6mm, 5μm, C8

Flow Rate: 1.0ml/Min

Temperature: 30°C

Volume: 10μl

Detector: PDA

Procedure: Inject 10μL of standard, sample into chromatographic system and measure the areas for the Ledipasvir and Sofosbuvirpeaks and calculate the % assay by using the formula

PREPARATION OF MOBILE PHASE [5]
Transfer 1000ml of HPLC water into 1000ml of beaker add 0.1%OPA.

Transfer the above solution 450ml of0.1%OPA, 550ml of Methanol is used as mobile phase. They are mixed and sonicated for 20min.

PREPARATION OF THE LEDIPASVIR AND SOFOSBUVIR STANDARD AND SAMPLE SOLUTION [5]
PREPARATION OF STANDARD SOLUTION:
Accurately weigh and transfer 90mg of Ledipasvir and 400mg Sofosbuvirinto 100ml of volumetric flask and add 10ml of Methanol and sonicate 10min (or) shake 5min and make with water. Transfers the above solution into 2.5ml into 25ml volumetric flask dilute to volume with water.

PREPARATION OF SAMPLE STOCK SOLUTION
Commercially available 20 tablets ware weighed and powdered the powdered equivalent to the 519.7mg of Ledipasvir and Sofosbuvir of active ingredients were transfer into a 100ml of volumetric flask and add 10ml of Methanol and sonicate 20 min (or) shake 10min and makeup with water.

	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1	Ledipasvir	3.303	5125885		1.11	15887
2	Sofosbuvir	7.303	8424190	20.23	1.08	10569

Transfers above solution 2.5ml into 25ml of the volumetric flask dilute the volume with Methanol. And the solution was filtered through 0.45μm filter before injecting into HPLC system.

METHOD VALIDATION [6–8]

SYSTEM SUITABILITY:
Tailing factor for the peaks due to Ledipasvir and Sofosbuvirin standard solution should not be more than 2.0.Theoretical plates for the Ledipasvir and Sofosbuvirpeaks in standard solution should not be less than 2000.

SPECIFICITY:
Specificity studies were carried for both pure drugs and drug product by comparing with blank and placebo. These blanks and placebo were compared with standard and sample shows that the analyte chromatographic peak is not attributable to more than one component as the impurities are not available.

Forced Degradation Studies [6]
In the present investigation, as there was no interference of impurities with the analyte peaks, forced degradation studies were conducted with the same LC conditions developed to separate drug peaks of interest from their degradants which proves the stability indicating power of the method. Intentional degradation was attempted to various stress conditions such as acid hydrolysis (using 1.0N HCl), base hydrolysis (using 1.0 N NaOH), oxidative hydrolysis (using 3.0%v/v H2O2), thermal degradation (heated at 70°C for 14 days) and photolytic degradation (to overall illumination of $\geq$210Wh/m2 at room temperature with UV light for 14 days), to evaluate the ability of the proposed method to separate Ledipasvir and Sofosbuvir from their degradation products. Acidic hydrolysis: Forced degradation in acidic media was performed by taking accurately weighed samples of 519.7mg of Ledipasvir and Sofosbuvir each in separate 5mL volumetric flasks. Then 2mL of 1N HCl was added, made to dissolve and final volume was made up to the mark with 1N HCl to get mg/mL solutions and these were kept at 70°C for 2 days and analyzed after suitable dilution.

Basic hydrolysis:
Forced degradation in basic media was performed by dissolving separately an accurately weighed quantities (122.5mg each) of Ledipasvir and Sofosbuvir in 1N NaOH in 5mL volumetric flasks and final volume was made up to 5mL with the same to get mg/mL solutions and these solutions were kept at 70°C for 2 days and analyzed after suitable dilution.

Oxidative degradation:
Oxidative degradation studies were carried out in 3% (v/v) H2O2. Stock solutions of 122.5mg/mL of Ledipasvir

and Sofosbuvir were prepared and kept at 70°C for 2 days and analyzed after suitable dilution. Photo Degradation: For photolytic stress, samples of drug substances in solid state were irradiated with UV radiation (overall illumination of $\geq$210Wh/m2 at room temperature with UV radiation), for 14 days. Stock solutions of 1mg/mL were prepared in methanol from the exposed drug substances individually.

Thermal Degradation:
For thermal stress, 122.5mg of samples of drug substances in solid state were packed in glass vials and placed in a controlled temperature oven at 70°C for 14 days. Stock solutions of 1mg/mL were prepared in methanol from the exposed drug substances individually. For HPLC analysis, all the stressed sample solutions were diluted with mobile phase to obtain final concentration of 60μg/mL of Ledipasvir and Sofosbuvir and 100μg/mL of Rabeprazole respectively. Similarly, mixture of both drugs in a concentration of 60μg/mL of Ledipasvir and Sofosbuvir and 100μg/mL of Rabeprazole was prepared prior to analysis by HPLC. Besides, solutions containing 60μg/mL of Ledipasvir and Sofosbuvir and 100μg/mL of Rabeprazole for each drug separately were also prepared without performing the degradation of both the drugs. Then 20μL of above solutions were injected into the HPLC system and analyzed.Solution of standard, sample, blank and placebo were prepared as per test procedure and injected into the HPLC system.

Acceptance criteria:
Chromatogram of standard and sample should be identical with near Retention time.

Blank interference
A study to establish the interference of blank was conducted. Diluent was injected into HPLC system as per the test procedure.

Acceptance criteria
Chromatogram of blank should not show any peak at the retention time of analyte peak. There is no interference due to blank at the retention time of analyte. Hence, the method is specific.

LINEARITY
Prepare a series of standard solutions and inject into HPLC system. Plot the graph of standard versus the actual concentration in μg/ml and determine the coefficient of correlation and basis for 100% response.

Acceptance criteria
Linearity regression coefficient of average peak area response of replicate injections plotted against respective concentration should not be less than 0.999. The % y-intercept as obtained from the linearity data (without extrapolation through origin 0, 0) should be within $\pm$2.0.

Statistical Evaluation

A graph between the concentration and the average area was plotted. Points for linearity were observed. Using the method of least squares, a line of best fit was taken and the correlation Coefficient, slope and, y-intercept were calculated.

PRECISION

Preparation of sample

- Transfer the 122.5mg of sample into a 100ml of volume at flask and add 10ml of water and 10ml of Methanol and sonicate 20min and makeup with water. Transfer the above solution into 2.5ml into 25ml volume metric flask dilute to the volume with water.
- The method precision parameters were evaluated from sample chromatograms obtained, by calculating the % RSD of peek areas from 6 replicate injection.

Acceptance criteria: The injection reproducibility requirements are met if the %RSD for peak areas is not more than 2.0 and for retention times is not more than 2.0.

RECOVERY/ACCURACY

Recovery study can be performed in the concentration range of 80% to 120% of the target concentration of the test. Minimum 3 concentrations are recommended.

Acceptance criteria:

The average percentage recovery was between 98-102% and Relative standard deviation of these recovery concentrations was less than 2%.

RESULTS AND DISCUSSION

SYSTEM SUITABILITY:

Table 1: System suitability data of Ledipasvir and Sofosbuvir

Parameter	Ledipasvir	Sofosbuvir	Acceptance criteria
Retention time	3.303	7.303	+-10
Theoretical plates	17053	10841	>2500
Tailing factor	1.10	1.07	<2.00
% RSD	0.7	0.8	<2.00

Standard Results of Ledipasvir

RESULT

Results of system suitability study are summarized in the above table. Six consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicate a good system for analysis.

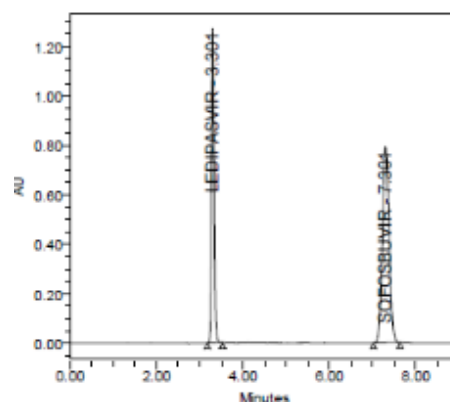| S.no	Sample name	RT	Area	USP plate count	USP tailing
1.	Injection1	3.301	5154154	15844	1.12
2.	Injection 2	3.308	5584697	16253	1.10
3.	Injection 3	3.304	4622291	16360	1.09
4.	Injection 4	3.301	3607569	15504	1.10
5.	Injection 5	3.304	3336067	15520	1.09
S.no	Sample name	RT	Area	USP plate count	USP tailing
1.	Injection 1	7.301	8518567	10496	1.08
2.	Injection 2	7.334	9220560	10564	1.08
3.	Injection 3	7.368	7640607	10781	1.07
4.	Injection 4	7.392	5932037	10553	1.06
5.	Injection 5	7.421	5512700	10416	1.06

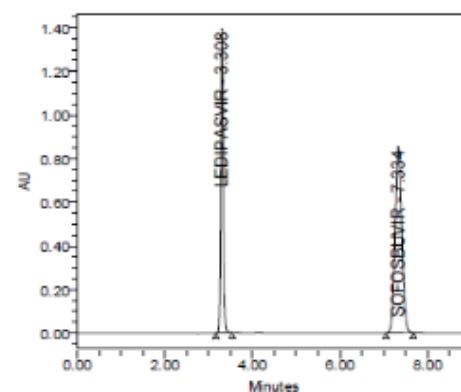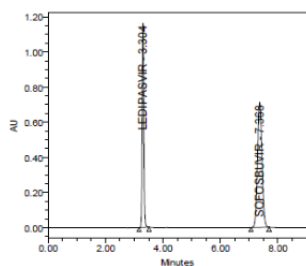**Fig 02:** Typical Chromatogram of Standard-2; Injection-1

Fig 03: Typical Chromatogram of Standard-2; Injection-2

Table 2: Specificity data for Ledipasvir and Sofosbuvir

S no	Sample name	Ledipasvirarea	Rt	Sofosbuvir Area	Rt
1	Standard	4460355	3.301	7364894	7.301
2	Sample	4460496	3.296	7363003	7.257
3	Blank	-	-	-	-
4	Placebo	-	-	-	-

Results of forced degradation study for Ledipasvir

Type of stress	Degradation products/ Drug (D)	Retention time	% Area	Peak purity	Result
Acidic Hydrolysis (mg/mL in 1N HCl) at 70ºC for 2 days	-	3.252	4236590	0.999	Passed
Basic Hydrolysis (mg/mL in 1N NaOH) at 70ºC for 2 days	-	3.265	4227521	0.999	Passed
Oxidative Hydrolysis (mg/mL in 3% v/v H_2O_2) at 70 ºC for 2 days	-	3.277	4299323	0.999	Passed
Photo Degradation (to UV light) for 14 days	-	3.287	4261372	0.999	Passed
Thermal Degradation at 70ºC for 14 days	-	3.287	4213277	0.999	Passed

Table 3: Results of forced degradation study for Sofosbuvir

Type of stress	Degradation products/ Drug (D)	Retention time	% Area	Peak purity	Result
Acidic Hydrolysis (mg/mL in 1N HCl) at 70ºC for 2 days	-	6.847	7039509	0.999	passed
Basic Hydrolysis (mg/mL in 1N NaOH) at 70ºC for 2 days	-	6.977	7081666	0.999 0.999	passed passed
Oxidative Hydrolysis (mg/mL in 3% v/v) at 70 ºc for 2 days	-	7.056	7045562	0.999	passed
Photo Degradation (to UV light) for 14 days	-	7.138	7066831	0.999	passed
Thermal Degradation at 70ºC for 14 days	-	7.113	7027361	0.999	passed

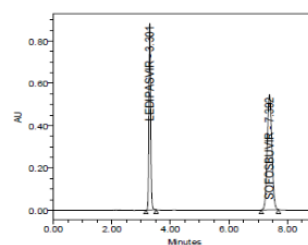**Fig 04:** Typical Chromatogram of Standard-2; Injection-3

Fig 05: Typical Chromatogram of Standard-2; Injection-4

SPECIFICITY:

Specificity data for Ledipasvir and Sofosbuvir

RESULT

The forced degradation study showed the method was highly specific, the chromatographic peaks does not interfere with any other impurities. This proves that, excipients have
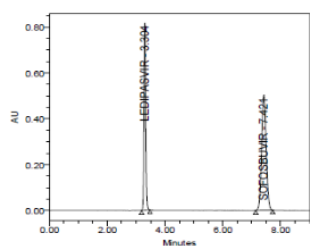
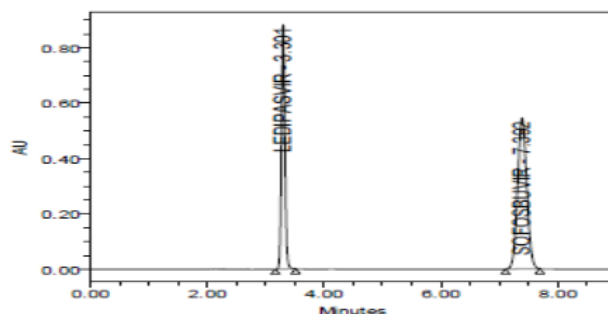**Fig 06:** Typical Chromatogram of Standard-2; Injection 5

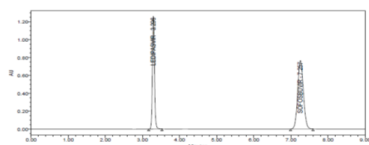**Fig 10:** Chromatogram representing specificity of standard

Fig 07: Typical Chromatogram of Sample-1
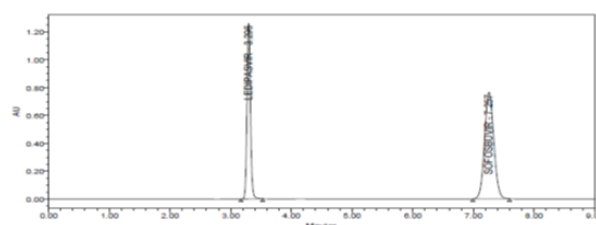System suitability chromatography of Ledipasvir and Sofosbuvir

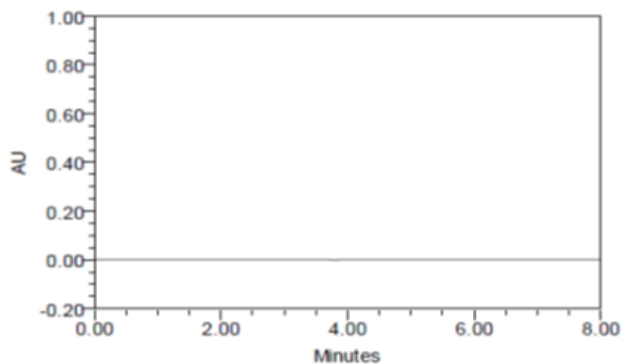**Fig 11:** chromatogram representing specificity of sample
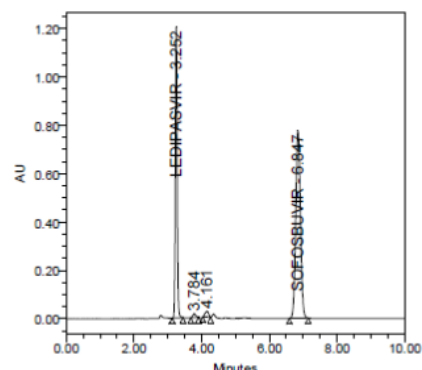

Fig 08: Typical chromatogram of the blank

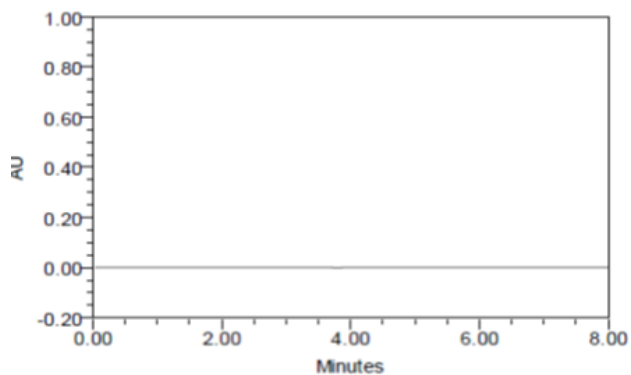**Fig 12:** Chromatograms of Acid stress treated Ledipasvir and Sofosbuvir mixture
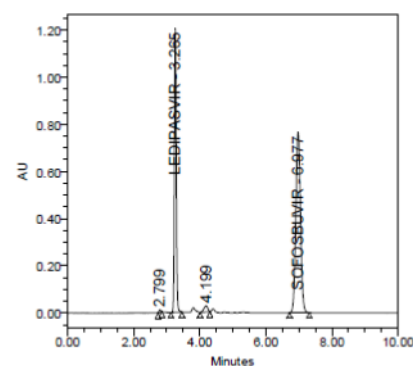

Fig 09: Typical chromatogram of the Placebo

Fig 13: Chromatograms of Base stress treated Ledipasvir and Sofosbuvirmixture

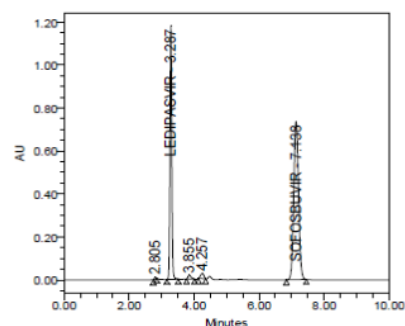**Fig 14:** Chromatograms of H2O2 stress treated Ledipasvir and Sofosbuvirmixture

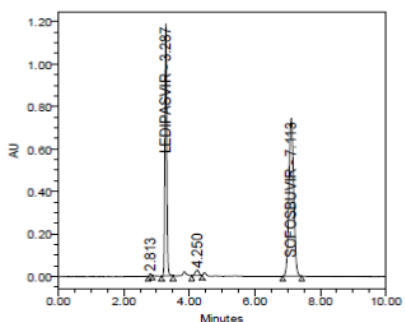**Fig 15:** Chromatograms of UV stress treated Ledipasvir and Sofosbuvirmixture

Fig 16: Chromatograms of Thermal stress treated Ledipasvir and Sofosbuvirmixture

Fig 17: Typical chromatogram for Accuracy 50 %

Fig 18: Typical chromatogram for Accuracy 100 %

Fig 19: Typical chromatogram for Accuracy 150 %

no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So the method is highly selective.

ACCURACY:

Accuracy data for Ledipasvir

RESULT

Results of accuracy study are presented in the above table. The measured value was obtained by recovery test. Spiked amount of both the drug were compared against the recovery amount.

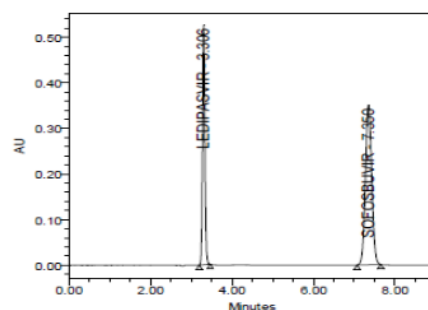% Recovery was 100.00% for Ledipasvirand 100.00% forSofosbuvir. All the results indicate that the method is highly accurate**.**

PRECISION:

Table 4: Accuracy data for Ledipasvir

S.NO	Accuracy Level	injection	Sample area	RT
1	50%	1	2234211	3.306
		2	2238492	3.306
		3	2237285	3.300
2	100%	1	4462488	3.304
		2	4466089	3.306
		3	4462709	3.305
3	150%	1	6693297	3.301
		2	6690064	3.300
		3	6695603	3.302

Table 5: Accuracy (%recovery) results of Ledipasvir

S.NO	Accuracy Level	Sample Name	Sample weight	μg/ml added	μg/ml found	% Recovery	% Mean
1	50%	1	61.25	44.550	44.67	100	
		2	61.25	44.550	44.76	100	100
		3	61.25	44.550	44.73	100	
2	100%	1	122.50	89.100	89.22	101	
		2	122.50	89.100	89.29	100	100
		3	122.50	89.100	89.23	100	
3	150%	1	183.75	133.650	133.82	100	
		2	183.75	133.650	133.76	100	100
		3	183.75	133.650	133.87	100	

Table 6: Accuracy data for Sofosbuvir

S.NO	Accuracy Level	injection	Sample area	RT
1	50%	1	3685084	7.350
		2	3684559	7.329
		3	3688142	7.308
2	100%	1	7362645	7.292
		2	7366522	7.292
		3	7362418	7.276
3	150%	1	11041980	7.264
		2	11047612	7.254
		3	11043057	7.247

Table 7: Accuracy (%recovery) results of Sofosbuvir

S.NO	Accuracy level	Sample Name	Sample weight	μg/ml added	μg/ml found	% Recovery	% Mean
1	50%	1	61.25	200.000	199.54	100	
		2	61.25	200.000	199.51	100	100
		3	61.25	200.000	199.71	100	
2	100%	1	122.50	400.000	398.68	100	
		2	122.50	400.000	398.89	100	100
		3	122.50	400.000	398.67	100	
3	150%	1	183.75	600.000	597.91	100	
		2	183.75	600.000	598.21	100	100
		3	183.75	600.000	597.97	100	

Table 8: Precision data for Ledipasvir

S.no	RT	Area	%Assay
injection1	3.320	4465231	99
injection2	3.321	4462350	99
injection3	3.316	4464645	100
injection4	3.312	4462083	99
injection5	3.313	4468154	100
injection6	3.312	4466897	99
Mean			99
Std. Dev.			0.18
% RSD			0.18

Fig 21: Chromatogram for precision injection 2

Table 9: Precision data for Sofosbuvir

S.no	RT	Area	%Assay
injection1	7.458	7360011	100
injection 2	7.471	7368755	100
injection 3	7.451	7364800	100
injection 4	7.419	7365230	100
injection 5	7.398	7361573	100
injection 6	7.370	7361600	100
Mean			100
Std. Dev.			0.12
%RSD			0.12

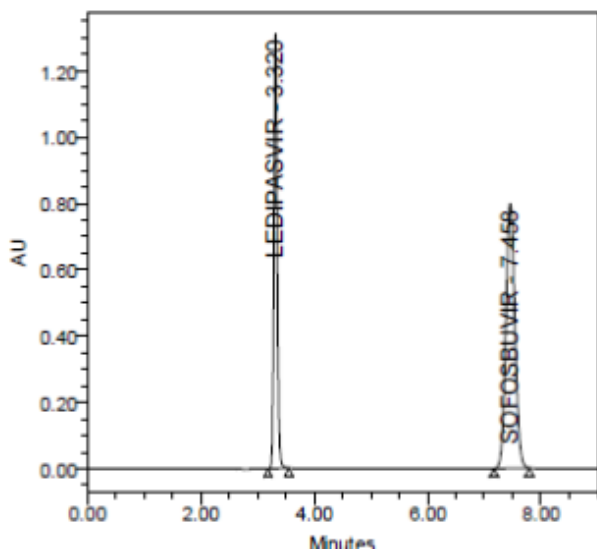**Fig 22:** Chromatogram for precision injection 3

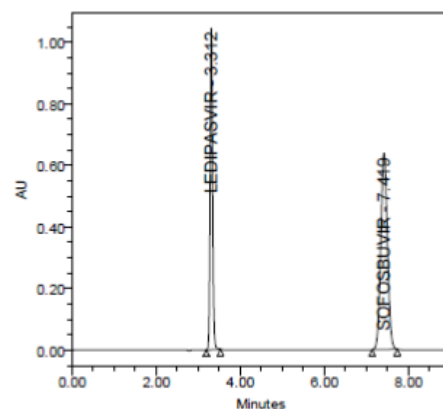**Fig 20:** Chromatogram for precision injection 1

Fig 23: Chromatogram for precision injection 4
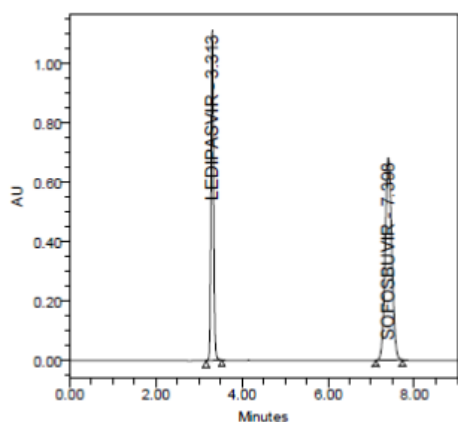
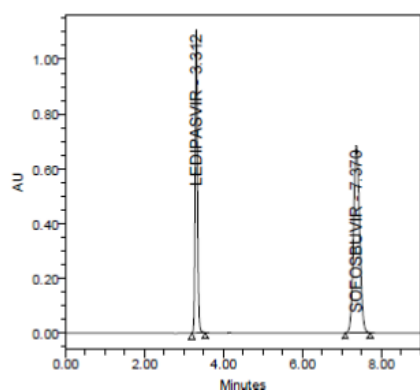**Fig 24:** Chromatogram for precision injection 5

Fig 25: Chromatogram for precision injection6

RESULT

Results of variability were summarized in the above table. % RSD of peak areas was calculated for various run. Percentage relative standard deviation (%RSD) was found to be less than 2% which proves that method is precise.

LINEARITY:

Table 10: Linearity data for Ledipasvir

s.no	Conc(μg/ml)	RT	Area
1.	50	3.297	2230184
2.	75	3.299	3347897
3.	100	3.297	4462463
4.	125	3.297	5577829
5.	150	3.302	6694287
Correlation coefficient (r2)			0.999

RESULT

A linear relationship between peak areas versus concentrations was observed for Ledipasvir and Sofosbuvirin the range of 50% to 150% of nominal concentration.
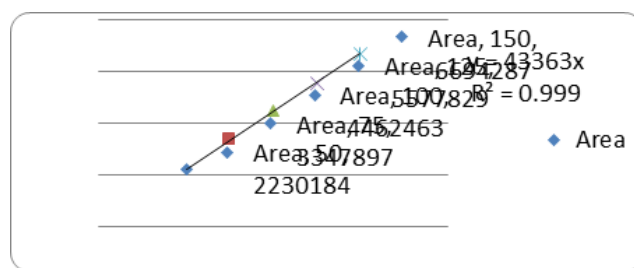

Fig 26: Linearity plot of Ledipasvir

Table 11: Linearity data for Sofosbuvir

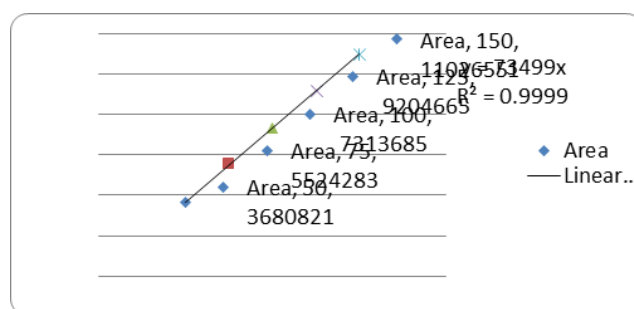| s.no	Conc(μg/ml)	RT	Area
1.	50	7.253	3680821
2.	75	7.246	5524283
3.	100	7.241	7363685
4.	125	7.228	9204665
5.	150	7.232	11026551
Correlation coefficient (r2)			0.999

Fig 27: Linearity plot of Sofosbuvir

Fig 28: Chromatogram representing linearity 1

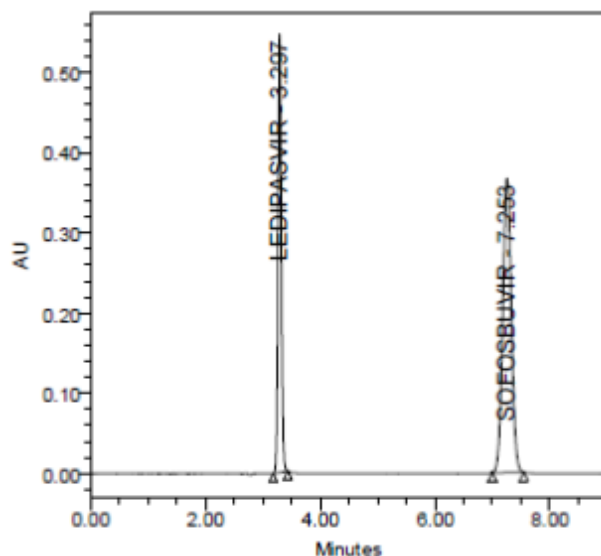**Fig 29:** Chromatogram representing linearity 2

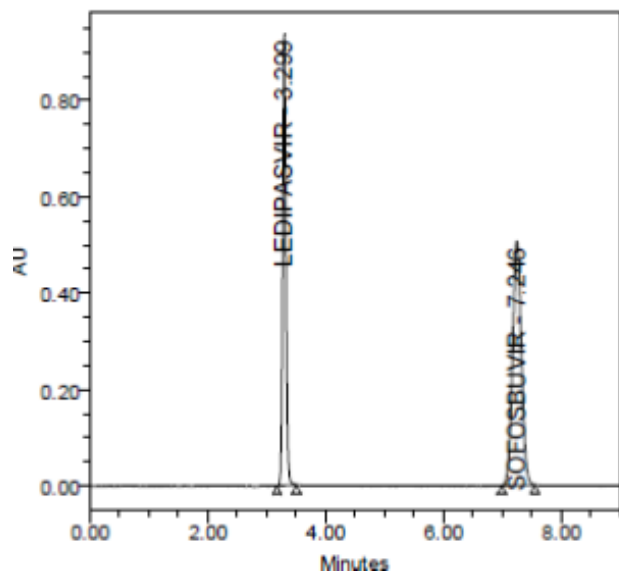**Fig 30:** Chromatogram representing linearity 3

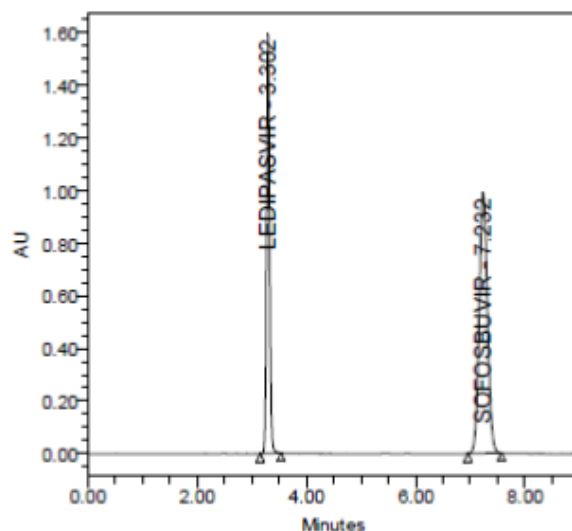**Fig 31:** Chromatogram representing linearity 4

Fig 32: Chromatogram representing linearity 5

Correlation coefficient was 0.999 for both Ledipasvir and Sofosbuvirwhich prove that the method is linear in the range of 50% to 150%.
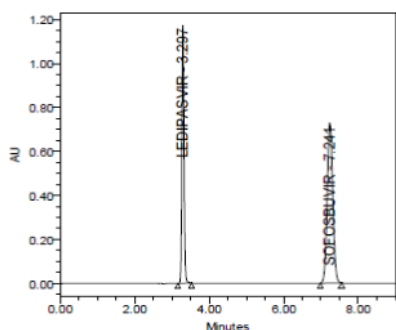
SUMMARY AND CONCLUSION

It was concluded that there was no stability indicating method reported for the above selected multi component dosage form, which promote to pursue the present work. The scope and objective of the present work is to develop and validate a new simple Stability Indicating RP-HPLC method for Ledipasvir and Sofosbuvirin bulk form. In simultaneous RP-HPLC method development, Waters HPLC with PDA detector and column used is Zorbax C8 (250 X 4.6mm) column with 5-micron particle size. Injection volume of 10 μL is injected and eluted with the mobile phase selected after optimization was 0.1% OPA and Methanol in the ratio of 45:55 was found to be ideal. The flow rate was found to be optimized at 1.0 mL/min. Detection was carried out at 236 nm. Quantitation was done by external standard method with the above mentioned optimized chromatographic condition. This system produced symmetric peak shape, good resolution and reasonable retention times of Ledipasvir and Sofosbuvir were found to be 3.303 and 7.303minutes respectively. The Ledipasvir and Sofosbuvir showed linearity in the range of 50-150μg/mL and 50-150μg/mL respectively. The slope and correlation coefficient values for Ledipasvir were found to be 43363 and 0.999respectivelyand 73499 and 0.999 respectively for Sofosbuvir which indicates excellent correlation between response factor Vs concentration of standard solutions. Precision of the developed method was studied under system precision and method precision. The %RSD values for precision was found to be within the acceptable limit, which revealed that the developed method was precise. The developed method was found to be robust.

The%RSD value for percentage recovery Ledipasvir and Sofosbuvir was found to be within the acceptance criteria. The results indicate satisfactory accuracy of method for simultaneous estimation of the Ledipasvir and Sofosbuvir. The forced degradation study showed the method was highly specific.

Conflict of Interest

Authors declared the no conflicts of interest.

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