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DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-HPLC METHOD FOR THE DETERMINATION OF TELAPREVIR IN RAW MATERIAL AND FINISHED PRODUCT

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Abstract:

Hepatitis C is a liver sickness caused by hepatitis C virus (HCV), which can be both acute and chronic in condition. Telaprevir is a Hepatitis C drug which acts as protease inhibitor that objective the viral HCV NS3-4A serine protease and disrupts processing of viral proteins and arrangement of a viral replication complex in Hepatitis C disease. A reversed-phase HPLC method was developed and validated for the determination of Telaprevir in raw material and to determine impurities and degradants that may developed in the tested samples. The separation was achieved on Waters C18 column (4.6 x 250 mm, 5µm) using mobile phase consisting of 65% Potassium Phosphate Dibasic Buffer (pH 7) and 35% Acetonitrile (100% ACN). The flow rate was 1.0 mL/min, injection volume 20µL, and detection was accomplished at 267 nm. The retention time for Telaprevir was 13 minutes. The developed method was validated and met all the acceptance criteria for validation parameters- system suitability, specificity, solution stability, robustness, linearity, accuracy, precision, limit of detection (LOD), and limit of quantitation (LOQ). The LOD was determined to be 0.1 ppm and LOQ was found to be 0.5 ppm.

Key words: *Telaprevir, Degradation, RP-HPLC, Validation and Finished product*

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INTRODUCTION:

Viral hepatitis can be brought on by various viruses, for example, hepatitis A, B, C, D and E[1].Hepatitis C is a liver sickness brought on by the hepatitis C infection, the infection can bring about both intense and endless hepatitis contamination, running in seriousness from a gentle disease enduring a couple of weeks to a serious, lifelong illness. Telaprevir is a hepatitis C virus (HCV) NS3/4A protease inhibitor, in combination with peg-interferon alfa and ribavirin, for the treatment of genotype 1 chronic hepatitis C (CHC)[4]. Chemical structure of Telaprevir is shown in the figure 2.Telaprevir is an orally accessible peptidomimetic drug with movement against hepatitis C infection (HCV). Telaprevir is a particular protease inhibitor that targets the viral HCV NS3-4A serine protease and disrupts processing of viral proteins and arrangement of a viral replication complex. Telaprevir, a tetra peptide mimetic, is HCV NS3/4A protease inhibitor. It is chemically known as (1S,3aR,6aS)-2-[(2S)-2-({(2S)-2-cyclohexyl-2-[(pyrazin-2-ylcarbonyl) amino] acetyl} amino)-3,3-dimethyl butanoyl]-N-[(3S)-1-(cyclopropylamino)-1,2-dioxohexan-3-yl]-3,3a,4,5,6,6a-hexa hydro-1H-cyclopenta[c]pyrrole-1-carboxamide. Its molecular formula is $C_{36}H_{53}N_7O_6$ and its molecular weight is 679.85. The primary purpose of this research project was to develop and to validate a simple HPLC method for determination of Telaprevir in the raw material and finished product. This is beneficial in any clinical environment where the concentration of Telaprevir is needed to understand any patient issues along with the Pharmaceutical industry to prepare the multiple steps that may be needed to prepare the raw material for production. There are very few analytical methods that have been reported for the determination of Telaprevir in Bulk and Formulations. Studying the stability of a drug and being able to monitor degradation products aids in the clinical treatments/early product development and shelf life for the drug. The present study was aimed at developing simple, specific, accurate and precise HPLC method for the determination of telaprevir in commercially available pharmaceutical formulations, based on direct UV-detection.

EXPERIMENTAL:

Quantitative HPLC was performed on the Waters Alliance 2695 Separations Module is a high performance liquid chromatographic system with a quaternary, low-pressure mixing pump and inline vacuum degassing. Flow rates from 50 uL/min to 5 mL/min can be generated for use with 2.1 mm ID columns and larger. The auto-sampler has a maximum capacity of 120 vials (12x32, 2-mL) with programmable temperature control from 4 to 40°C. A heated column compartment provides temperatures from 5 degrees above ambient to 65°C. The detector is a photodiode array (model 2996) with a wavelength range of 190-800 nm and sensitivity settings from 0.0001-2.0000 absorbance units. X-Terra RP-C18 Column (250x4.6 mm i.d; particle size 5 µm) was used. The HPLC system was equipped with Empower-2 solution software.

Chemicals and reagents: Standard drug Telaprevir was obtained as working standard from Mylan laboratories Hyderabad. HPLC grade acetonitrile, and hydrochloric acid were procured from Merck Ltd., India. Analytical grade sodium hydroxide, hydrogen peroxide and other chemicals used in the study were procured from CDH chemicals Ltd, Mumbai, India.

Preparation of standard solutions: Telaprevir standard solution (6000ppm): Weight accurately 150 mg of telaprevir and transfer in to 25 mL volumetric flask. Add solvent (75% ACN) and shake gently; sonicate the solution till completely dissolved. Make up the volume with diluting solvent (75% ACN) which produces 6000ppm stock solution. Transfer 1.6 mL from stock solution into 10 mL volumetric flask, make up the volume with solvent and shake gently to get 1000 ppm telaprevir solution.

Selection of stationary phase: For the telaprevir research work six different columns were cleaned according to the procedure mentioned above and sample injected was 1000ppm telaprevir solution, prepared from 6000ppm stock solution. Considering ICH criteria of the column, above 6 columns were tested. Column 1 has good tailing factor and theoretical plates but it has irreproducible results, whereas column 2 and column 3 had high tailing factor 1.694and 1.809. column 4 also producing irreproducible results with extra small peaks, whereas column 5 had less tailing factor and front tailing too. Finally, column 6 belongs to Waters brand with serial no# WAT054275 provided acceptable tailing factor 1.004, retention time 13.195 and theoretical plates 11470. So that Column 6 was selected for the method development and validation of Telaprevir.

Selection of mobile phase: In reverse phase HPLC, peak tailing is frequently occurring due to the interaction between protonated amine functional group in ketoamide moiety of telaprevir and an acid silanol group present in octadecyl silica surface present in stationary phase. After several trials, the peak tailing telaprevr triggered us to adjust the mobile phase pH-7 with dibasic potassium phosphate buffer. The neutral pH-7 prevents the protonation of amine functional group in ketoamide moiety of telaprevir and thereby inhibits the interaction with silanol group which reduces the peak tailing factor. Different proportions of acetonitrile and water with

pH adjusted to 7 by using dibasic phosphate buffer steadily improved the chromatographic behavior. Optimum mobile phase composition was obtained after several trials as acetonitrile: phosphate buffer (di basic) pH=7 at the proportion of 35:65 (v/v) at flow rate of 1.0 mL/min is a suitable mixture for separation of telaprevir.

Selection of Detection wavelength: The proper wavelength was needed to determine maximum detector response. The maximum UV response of telaprevir for detection and quantification was determined by using Hitachi U-29100 UV-VIS spectrophotometer. The first step was to run a UV-spectrum (from 190-600 nm) using an HPLC system equipped with the Diode Array Detector. The resulting spectrum depicts that the telaprevir absorbs maximum light at 260 nm to 270 nm in 75 % Acetonitrile as diluent. The longer wavelength of 267 nm was selected since it produces less noise, which minimizes problems that may exhibit around the active ingredient when attempting to quantify Telaprevir.

Preparation of Linearity dilutions: In this study, for the determination of Nominal concentration various increasing concentrations of Telaprevir were prepared from the Telaprevir stock solution 6000 ppm.5 different concentrations ranges from 100 to 1500ppm were prepared from 6000 ppm. Table 6 shows the volume of stock solution used to make the sample final volume of 10ml of 5 different solutions. After desired volume of stock solution is added to 10ml of volumetric flask then make up the volume with solvent up to 10ml.

Table 1: Preparation of five concentrations of Telaprevir used in the determination of nominal concentration.

Concentration of Telaprevir (ppm)	Volume of 6000 ppm Stock Solution (mL)	Flask Volume (mL)
100	0.166	10.0
500	0.833	10.0
750	1.25	10.0
1000	1.66	10.0
1500	2.5	10.0

Acid stress study: A 2.5 mL of 3M hydrochloric acid solution was added to 2.5 mL solution of Telaprevir Stock solution (6000 ppm). The solution was heated at 75°C for 24 hours. It was then neutralized with 2.5 mL of 3M NaOH, then diluted to 10.0 mL with the diluting solvent and mixed well. Finally inject it into the HPLC and areas were recorded.

Base Stress studies: A 2.5 mL of 3M sodium hydroxide was added to 2.5 mL solution of Telaprevir stock solution (6000 ppm). The solution was heated at 75°C for 1 and 7days. It was then neutralized with 2.5 mL of 3m HCl and diluted to 10.0 mL with the diluting solvent and mixed well.

Oxidation Stress studies: A 2.5ml of 3% hydrogen peroxide solution was added to 2.5 mL solution of Telaprevir stock solution (6000ppm) and heated at 75°C for 1 day. The drug was very sensitive towards hydrogen peroxide and underwent complete degradation. As a result, stability of Telaprevir towards hydrogen peroxide was studied under milder conditions. So that the concentrations of hydrogen peroxide decreased a lot to get the desired degradation between 5-10%.

Heat Stress studies: To determine stability of Telaprevir towards heat, three samples of 2.5 mL of Telaprevir stock solution (6000ppm) were heated for 24hours, 12hours, 10hours at 75°C separately. The solutions were then cooled to room temperature and diluted to 10.0 mL with the diluting solvent. They were mixed well and injected into the HPLC system.

UV sensitivity Study: UV degradation studies are carried out by taking the raw telaprevir drug in to a petri plate and kept under the UV light for 3 days. Meanwhile, after 24 hours and 72 hours 10mg of telaprevir drug is taken by which 1000ppm of telaprevir solution is prepared and injected in to the hplc and areas were recorded.

Mixed Degradation Study: Here in this study 0.5%H2O2 and 0.1M Hcl is mixed for the aim of of this study is to check the chromatographic conditions which are developed can separate the active ingredients from its degradants are not. If not, solvent optimization is required to separate active ingredients and its degradants.

RESULTS AND DISCUSSION:

Optimized Chromatographic Conditions: A reversed phase isocratic HPLC stability-indicating method was developed for the determination of Telaprevirand a reversed phase gradient elution technique is developed for the separation of degradants using reversed-phase liquid chromatography. (a) The optimum conditions used

for the developed method for the determination of telaprevir are as follows:

- Elution Technique: Isocratic (reversed-phase separation)
- HPLC: 1100 Series HPLC System with MWD (UV/VIS Detector), Agilent Technologies.
- Column: Waters C 18 5µ 4.6mm x 250mm column
- Mobile Phase: Mobile Phase A.25mM Potassium Dibasic Phosphate Buffer pH 7; B.100% ACN.
- Solvent Strength: 65% Buffer (phosphate pH 7) and 35% ACN.
- Absorbance: 267 nm
- Flow rate: 1.0 mL/min.
- Injection Volume: 20µL
- Run time: 20 minutes.

(b) The optimum conditions used for the developed method for the separation of telaprevir degradants are as follows:

- Elution Technique: Gradient (reversed-phase separation)
- HPLC: 1100 Series HPLC System with MWD (UV/VIS Detector), Agilent Technologies.
- Column: Waters C 18 5µ 4.6mm x 250mm column
- Mobile Phase: 5-90% ACN for 40 minutes {mobile phase A: buffer (phosphate buffer pH 7), mobile phase B: ACN 100%}
- Absorbance: 267 nm
- Flow rate: 1.0 mL/min.
- Injection Volume: 20µL
- Run time: 40minutes

Validation Parameters: System suitability: The prepared standard solutions of 1000 µg/ml Telaprevir were injected into the HPLC system under the established optimum separation conditions. Standard solution 1 was injected six times, and standard solution 2 was injected twice. The % RSD values for retention times and peak areas of six replicate injections for Telaprevir standard 1 and two replicate injections for Telaprevir standard 2 were 0.222% and 0.037% respectively. The % Drift, which was calculated using the following equation, is less than 2%. A_S is average peak area of six replicate standard injection and A_C is the peak area of check standard. The tailing factor for Telaprevir peak is less than 2.0 and more than 0.9 and the number of theoretical plates are more than 2000. These results fulfilled the required system suitability acceptance criteria. Table 14 shows the results.

Table-2: System suitability parameters for Telaprevir

STD 1 INJ #		Peak Areas	Retention times	Plate count	Tailing factor
1.	1000ppm	18254.6	13.373	5358	1.028
2.	1000ppm	18223.7	13.282	5126	1.026
3.	1000ppm	18253.8	13.354	5103	1.021
4.	1000ppm	18204.7	13.371	4891	1.034
5.	1000ppm	18202.6	13.285	4901	1.037
6.	1000ppm	18145.9	13.280	4825	1.049

Solution Stability of Telaprevir: Telaprevir solution was injected immediately and then after 24, 48 and 72 hours of sample preparation, simultaneously peak areas were recorded for all the samples injected. All the 3 peak areas were compared with the immediately injected sample peak area and percentage change was calculated. The acceptance criteria for percentage change should be less than %2. Table 15 shows the results for the solution stability.

Table 3: Solution stability of Telaprevir over a period of 72 hours.

Time (hours)	Peak Area	% Change
0	18347.3	-
24	18341.1	0.033
48	18121.4	1.23
72	18082.1	1.44

Specificity: Determination of Telaprevir Peak Purity:

Once confirmed that the potential degradants are completely resolved from the Telaprevir peak, the next attempt was to ensure that the Telaprevir chromatographic peak in the force degraded sample is pure. For that, the degradation samples are mixed and used to test specificity to show that Telaprevir is pure and separated from their degradants. This sample was injected in an HPLC instrument equipped with a Diode Array Detector (DAD). Peak purity tests were performed on Agilent 1200 HPLC system equipped with Diode Array Detector (DAD). Under the optimum separation conditions, the resulted purity factor is within the threshold limits. Validity of these results was further supported by the three-dimension plot of Telaprevir chromatographic peak (Figures 2-a and 2-b).

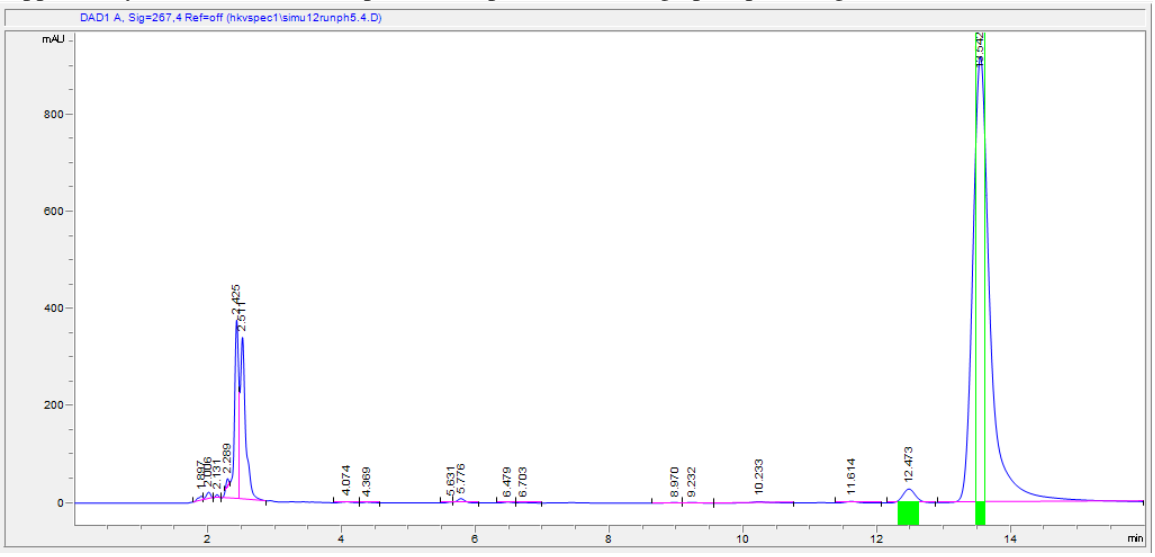


Figure 2-a: Peak purity of Telaprevir on Agilent 1200 HPLC-DAD

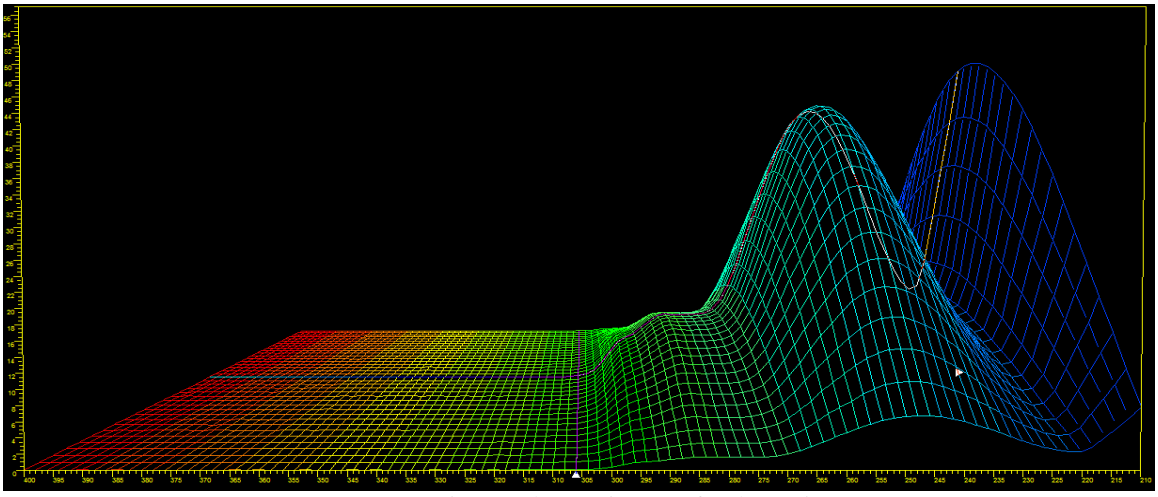


Figure 2-b: 3-D image of Telaprevir

Robustness Studies on Telaprevir Raw Material: The impact of deliberately changing the optimized chromatographic conditions on the retention and resolution of Telaprevir peak was investigated. In consideration of FDA guidelines these chromatograms are satisfied by peak tailing and theoretical plates. In addition, in no cases the peak shape distorted or does the peak interfere with the peaks of other sample components in the chromatogram. In summary, parameters studied for robustness did not display any potential interference in the determination of Telaprevir in the raw material when deliberate changes in the separation parameters were made. The results of robustness studies are summarized in Table –4

Table-4: Robustness study of Telaprevir

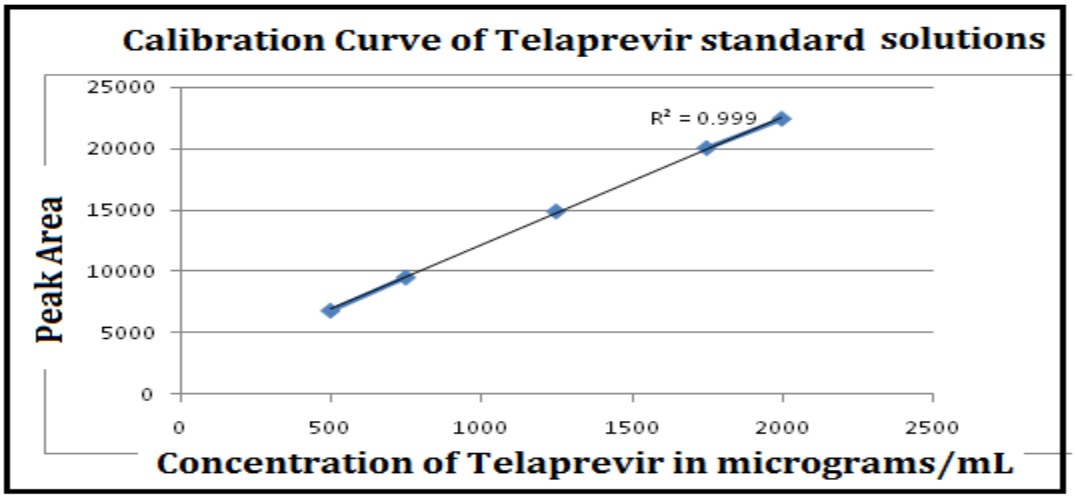
Experimental Conditions	Variation	Tailing factor	Theoretical plates
Buffer pH	7.2	1.649	230414
	7.0 (Nominal)	1.668	229932
	6.8	1.675	222297
Wavelength (nm)	262	1.921	163064
	267 (Nominal)	1.801	183718
	272	1.959	162906
Flow rate (mL/min)	1.2	1.567	221081
	1.0 (Nominal)	1.669	268861
	0.8	1.611	211635
Solvent strength (%B)	40	1.524	199855
	35	1.577	220544
	30	1.645	249715

Linearity: The linearity range tested for Telaprevir raw material was between 500 ppm and 2000 ppm. Solutions with different concentrations of Telaprevir were prepared as shown in Table 5 and injected into the HPLC system. The obtained peak areas for the corresponding concentrations of Telaprevir are summarized in Table-5

Table-5: Linearity Studies of Telaprevir in raw material

Concentration Level (ppm)	Volume of Stock Solution (mL)	Final Volume (mL)	Peak area
500	0.833	10	6856.2
750	1.25	10	9552.3
1250	2.083	10	14911.7
1750	2.916	10	20059.2
2000	3.333	10	22429.9

Figure-2: Calibration Curve of Telaprevir standard solutions in linearity study



Accuracy: The accuracy of the developed method was tested to determine closeness of the measured value to the true value. Accuracy of the developed method was studied by evaluating the recovery of Telaprevir from spike solutions. The recovery of Telaprevir at 80, 100 and 120% of the nominal concentration.The 6000 ppm stock solution was used. Table 21 show the preparation of the concentrations from stock solution.

Precision: The ICH guidelines recommend that repeatability should be assessed using a minimum nine determinations covering the specified range for the procedure (i.e., 3 concentrations and 3 replicates of each concentration (or) using minimum of six determinations at 100% of test concentration.

Table 6: Summary of Accuracy studies results (Raw Material)

	Accuracy results of Telaprevir			
Sample Preparation	Concentration (ppm)	Peak Area	Average Peak Area	Recovery
1	1250	14238.7	14216.93	99.11%
2		14251		
3		14161.1		
1	1750	18756	18718.26	99.29%
2		18689.8		
3		18709		
1	2000	21273	21224.53	98.74%
2		21437		
3		20963.1		

Instrument Precision (Injection Repeatability): Repeatability of injection of Telaprevir was determined by preparing one solution at 1500ppm of nominal concentration and carrying out six repeated injections.The precision of peak areas of six injections was calculated as relative standard deviation (%RSD). This analysis precision met the defined acceptance criteria of maximum allowable %RSD of NMT 1 %. Then calculate precision and %RSD. The results can be seen in the Table-4. Method precision was demonstrated by calculating % RSD of six independent preparations of 100% target concentration of 1250ppm of Telaprevir. It was also calculated for the impurities of six independent preparations of 1.250 ppm and all these were injected into hplc under developed method conditions. The results can be seen in the Table-6

Table-7(a): Peak area results of six injections of Telaprevir for injection precision.

Injection	Peak Area	Mean	Standard Deviation	Peak Area %RSD
1	18014.4	17929.15	81.675	0.45%
2	18059.9			
3	178902.7			
4	17820.7			
5	17898.3			
6	17888.9			

Table 7(b): Summary of Method Precision (Raw Material)

Injection #	Peak Area	Mean	Standard Deviation	Peak Area %RSD
1	17444.3	17496.98	190.75	1.09%
2	17684.5			
3	17758.3			
4	17245.4			
5	17393			
6	17456.1			

Limit of detection (LOD): The LOD is generally expressed as the concentration of the analyte sample (i.e., in ppm or in percentage). The detection limit of the developed method is the lowest analyte concentration that can produce a response detectable above the noise level of a system, typically, three times the noise level (3:1) .
The Limit of Detection (LOD) was evaluated over the course of 10 independent preparations of low concentrations of Telaprevir Reference Standard. All these 10 solutions are prepared from the stock solution of 5ppm, Table 30shows the preparations volume of stock solution. The cut off criteria was when signal to noise ratio exhibits a 3:1 ratio, which was found at 0.25ppm. Table 31displays the summarized results.

Table 8: Signal-to-Noise ratios of various concentrations of Telaprevir for LOD and LOQ.

Concentration of telaprevir in ppm	**Volume of 5ppm Stock Solution (mL)**	**Flask Volume (mL)**	Signal to Noise ratio
0.1	0.2	10.0	1.6
0.2	0.4	10.0	2.8
0.25	0.5	10.0	3.2
0.50	1	10.0	11.2
0.75	1.5	10.0	13.7
1.0	2	10.0	25.4
1.5	3	10.0	37.6
2	4	10.0	58.1
2.5	5	10.0	83.3

The quantification limit is the lowest level of analyte that can be accurately and precisely measured. Generally, Limit of Detection and Limit of quantification were studied at the same time.The limit of quantitation was evaluated over the course of six injections of low concentrations of Telaprevir Reference Standard. The cut off criteria is when signal to noise ratio exhibits a 10:1, which was produced at 1.2ppm. This concentration was injected 6 times and %RSD were calculated which should be less than or equal to 10%. Table 32 shows the results of 6 injections.

Table-9: Summary of Limit of Quantitation (LOQ)

Injections	Peak Area	Peak Area %RSD
1	61.4	5.40%
2	52.7	
3	58.3	
4	57.9	
5	56.3	
6	60.5	

Acid stress study: The following table 8 shows the different concentrations of Hcl and their degradation time and peak areas.

Table 10: Acid degradation study of different concentrations of Telaprevir under optimized chromatographic conditions.

Stress Condition	**Time Heated**	**Temperature**	**Area**	**Degradation**
CONTROLLED	-	-	18778.4	-
(A) 3M HCL	24 HRS	75^0C	8767.3	53.3
(B) 2M HCL	24 HRS	75^0C	9287.6	50.5
(C) 1M HCL	24 HRS	75^0C	15258.1	18.7
(D) 0.1M HCL	24 HRS	75^0C	15386.5	18.06
(E) 0.1M HCL	**1 HR**	**75^0C**	**17453.8**	**7.05**

Base stress study: The following table 9 shows the different concentrations of NaoH and their degradation time and peak areas.

Table 11: Base degradation study of different concentrations of Telaprevir under optimized chromatographic conditions.

Stress Condition	Time Heated	Temperature	Peak Area	Degradation
CONTROLLED	-	-	18778.4	-
(F)3M NAOH	24 HRS	75^0C	4857	74
(G)2M NAOH	24 HRS	75^0C	8041.4	57.1
(H)1M NAOH	24 HRS	75^0C	13752	27
(I)0.1M NAOH	24 HRS	75^0C	14129.8	24.7
(J)0.1M NAOH	**1 HR**	**75^0C**	**17283.8**	**7.9**

Oxidative stress study: The resulted chromatograms are shown in Figure 13 and the degradation results are given in Table 10.

Table 12: Oxidation study of different concentrations of Telaprevir under optimized chromatographic conditions.

Stress Condition	Time Heated	Temperature	Area	Degradation (%)
CONTROLLED	-	-	18164.3	-
(A) 3% H_2O_2	24 HRS	75^0C	81.6	9.09
(B) 2% H_2O_2	24 HRS	75^0C	299.5	14.5
(C) 1% H_2O_2	24 HRS	75^0C	3221.9	19.4
(D) 0.5% H_2O_2	24 HRS	75^0C	4269	33.3
(E) 0.1% H_2O_2	24 HRS	75^0C	12114	76.4
(F) 0.1% H_2O_2	10 HRS	75^0C	14624	82.2
(G) 0.1% H_2O_2	0 HRS	75^0C	15523.7	98.3
(H) 0.01% H_2O_2	0 HRS	-	16512.3	99.5

Thermal degradation study: The resulted chromatograms are shown in Figure 14, and the degradation results are given in Table 11.

Table 13: Heat stress results of Telaprevir under optimized chromatographic conditions.

Stress Condition	Time Heated	Temperature	Area	Degradation (%)
CONTROLLED	-	-	18164.3	-
(A)1000 ppm	10 HRS	75^0C	16438.1	9.5%
(B)1000ppm	12 HRS	75^0C	15848.4	12.7%
(C)1000ppm	24 HRS	75^0C	10655.7	41.3%

UV-Senstivity study: Telaprevir raw drug is stable against UV light. The resulted chromatograms were shown in the Figure 15.

Table 14: UV results of Telaprevir under optimized chromatographic conditions.

Stress Condition	Under UV light	Temperature	Area	Degradation (%)
CONTROLLED	-	-	18164.3	-
(A)1000 ppm	24 HRS	Ambient	18135.1	0.1%
(B)1000ppm	72 HRS	Ambient	16660.6	8.2%

CONCLUSION:

A stability indicating reversed phase hplc method has been developed and validated for Telaprevir, a Hepatitis C drug. An isocratic elution technique is used for the method development and validation. The run time of average 13 minutes is found in this method development and the mobile phase used was ACN and dibasic potassium phosate at a pH 7.0 at a ratio of 35:65. In Force degradation, Due to the formation of degradants with the Telaprevir its separation with the degradants was hard. Seperation of degradants from telaprevir was carried out by developing the Gradient elution method and the conditions.

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