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ANALYTICAL METHOD DEVELOPMENT, VALIDATION AND STRESS DEGRADATION STUDY OF DACLACTASVIR BY RP-HPLC METHOD

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ABSTRACT

To develop and validate simple, rapid, linear, accurate, precise and economical reverse phase-high-performance liquid chromatography (RP-HPLC) method for of stress degradation study of Daclatasvir. The separation and quantization were achieved on Inertsil C18(250 mm × 4.6ID,5 um). The mobile phase selected was Methanol: Water (80:20) at a flow rate of 0.8 ml/min and detection of analyte was carried out at 317nm at pH 3. The method exhibited good linearity over the range of 10–50 µg/mL. The drug is freely soluble in organic solvents Methanol. The drug was identified in terms of solubility studies and on the basis of melting point done by capillary tube method. The drug which when subjected to thermal, photolytic, oxidative, and acidic stress degraded into many degradation products. In most of the cases, the degradation rate was seen to be directly proportional to the amount of stress applied. The thermal stress was increased by increasing the incubation temperature, the faster the degradation took place. The values of LOD were found to be 0.845ug/ml for DCV. and the calculated LOQ values were found to be 0.054ug/ml. The low values of LOD and LOQ indicates the sensitivity of the method.

KEYWORDS: Daclatasvir, Method development, Validation, Force degradation, RP-HPLC.

INTRODUCTION

Validation[1,2,3]

Validation is an integral part of quality assurance which helps to maintain current good manufacturing practices which results in safety, quality, purity and efficacy of product. According to WHO "Validation is establishing documented evidence, which provides a high degree of assurance that a specific process will consistently produce products which meet its predetermined specifications and quality characteristics."

The documented act of demonstrating that any procedure, process and activity will consistently lead to the expected results. It also includes the qualification of systems and equipment. Manufacturers should plan validation in a manner that will ensure regulatory compliance and ensuring that product quality, safety and consistency are not compromised.

Analytical method validation[4,5,6,7]

The validation of analytical method is the process in determining the suitability of a given methodology by laboratory studies; that the method can meet the requirements for intended use. Method validation is not simply a measure of procedure but method validation is a measure of performance of the total analytical system.

According to USP "Validation is the process of providing documented evidence that the method does what it is intended to do." In other words, the process of method validation ensures that the proposed analytical methodology is accurate, specific, reproducible, and rugged for its intended use. Method validation is a regulatory requirement.

Various Guidelines describe typical analytical performance characteristics, how they are determined, and which subset of data is required to demonstrate validity, based on the methods intended use. These analytical performance characteristics are:

- Accuracy
- Precision
- Specificity
- Limit of Detection (LOD)/ Detection Limit (DL)
- Limit of Quantitation (LOQ)/ Quantitation Limit (QL)
- Linearity and range
- Ruggedness
- Robustness

Chromatography[8]

Chromatography is a non-destructive procedure for resolving a multi-component mixture into individual fractions. Quantitative analysis is carried out by measuring the area of the chromatographic peak. Hence chromatography can be used for qualitative and quantitative analysis. Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases that is mobile phase and stationary phase. Chromatography is mainly divided into two categories:

- **Adsorption chromatography:** Separation is mainly due to the interaction between solute and surface on the adsorbent. In this, stationary phase is solid and mobile phase is liquid. e.g.: TLC, HPTLC and GC.
- **Partition chromatography:** Separation is based on the partition coefficient of two phases. In this mode, both stationary phase and mobile phase are liquids e.g.: HPLC, GLC and PC.

Regulatory basis of Stability-Indicating Assays and Forced degradation studies[9,10]

The ICH guidelines have been incorporated as law in the Europe, Japan and in the United States, but in reality, besides these, other countries are also using them. A stability indicating profile that provides assurance on detection of changes in identity, purity and potency of the product has been put on the manufacturer. The expiry date should be based on real-time/real-temperature data. However, it is suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions.

Forced degradation

Since the conditions that cause instability and result in degradation products of the API cannot be predicted initially, one has to subject the API to a variety of stress conditions. Trial and error are needed to find the proper combination of stress agent concentration and time to effect degradation, preferably in the 5-15 % range. Depending on the API, not every stress agent may affect degradation, but each agent has to be evaluated to determine whether degradation occurs or not.

Typical degradative conditions involve hydrolysis, photolysis, acid/base reactions, and temperature. Achieving 100 % degradation would require too much effort and could be possibly cause secondary degradation. Secondary degradation products are the degradation products of the degradation products, which are not likely to be formed under normal storage conditions. Depending on the API, not all of the degradation conditions effect degradation,

and after a reasonable effort (varying concentrations and time) to produce a degradation product with no success, one can move on to the next condition.

MATERIALS

Table 1: Active drug.

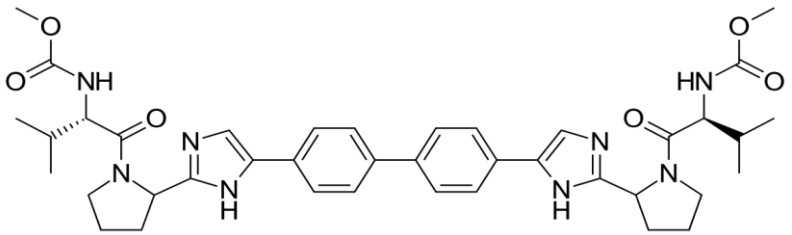
Name of drug	**Daclatasvir:** It is a new oral, direct-acting antiviral with potent pan genotypic activity. It is an inhibitor of hepatitis C virus (HCV) NS5A protein. NS5A is involved in both viral RNA replication and virus particle assembly.
Structure	
Molecular formula	$C_{40}H_{50}N_8O_6$
Category	Antivirus
Solubility	DMSO (Slightly), Methanol (Slightly)
Melting point	163^{o} - $165^{o}C$
Dose	In adults, Daclatasvir is orally administered at a dosage of 30 mg twice daily.
Uses	Daclatasvir is used with another antiviral medication (sofosbuvir) to treat chronic (long-lasting) hepatitis C, a viral infection of the liver.

Table 2: Solvents and Chemicals.

Sr. no.	**Solvents and Chemicals**	**Name of company**
1.	Methanol HPLC Grade	E. Merck Ltd., Mumbai, India
2.	Water HPLC Grade	E. Merck Ltd., Mumbai, India
3.	O – phosphoric acid AR Grade	E. Merck Ltd., Mumbai, India

Table 3: Marketed formulation.

Sr. no.	**Marketed Formulation**	**Manufacturer**
1.	Daclakem (Daclatasvir 60 mg tablet)	Alkem Laboratories Ltd., Mumbai, Maharashtra.

Table 4: List of equipment.

Sr. no	**Equipment**	**Make/ Model**
1	HPLC	Shimadzu LC2010
2	Detector system	UV detector
3	Analytical column	4.6 × 250 C-18 (inertsil)
4	Software	LC Solution
5	Precision balance	Mettler Tolledo
6	pH meter	Labinda
7	Grade ‘A’ certified Glassware	Borosil
8	Ultrasonicator	FAST CLEAN Ultrasonic Cleaner
9	Electronic balance	Electrolex New

RESULTS AND DISCUSSION

❖ Development of chromatographic validation method for estimation of drug

The standard solution of Daclatasvir was used for HPLC method development to determine Daclatasvir in presence of degraded products. Degraded samples were prepared by systematic forced degradation study. These samples were used for method development trials to optimize the method as a stability indicating method.

➢ Selection of mobile phase

The pure drug of Daclatasvir was injected into the HPLC system and run in different solvent system. Each mobile phase was allowed to equilibrate with stationary phase until steady base line was obtained. Different mobile phase like methanol and water, Acetonitrile and water in various proportions were tried. Different individual solvents as well as combination of solvent were tried to get a stable peak each mobile phase was filtered through 5 µm membrane filter and sonicated on ultra sonic bath. After trials methanol: water (80:20v/v) was found to be most satisfactory since it gave sharp peak with symmetry within limits and significant reproducible retention time.

➢ Selection of mobile phase

MeOH: WATER (80:20V/V)

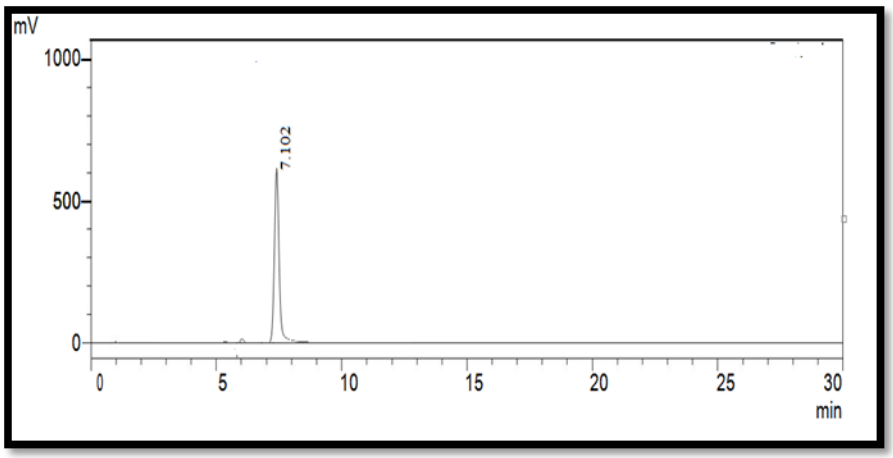


Fig. 1: Chromatogram of standard drug DCV (10 µg/ml).

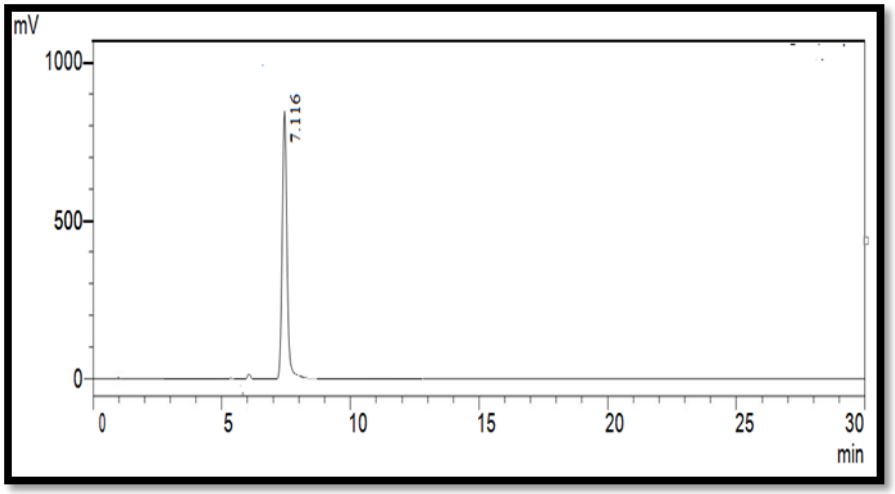


Fig. 2: Chromatogram of tablet DCV (10 µg/ml).

All the parameters of system suitability were observed within the acceptable limits as per the ICH guidelines for DCV hence system is suitable for the further validation.

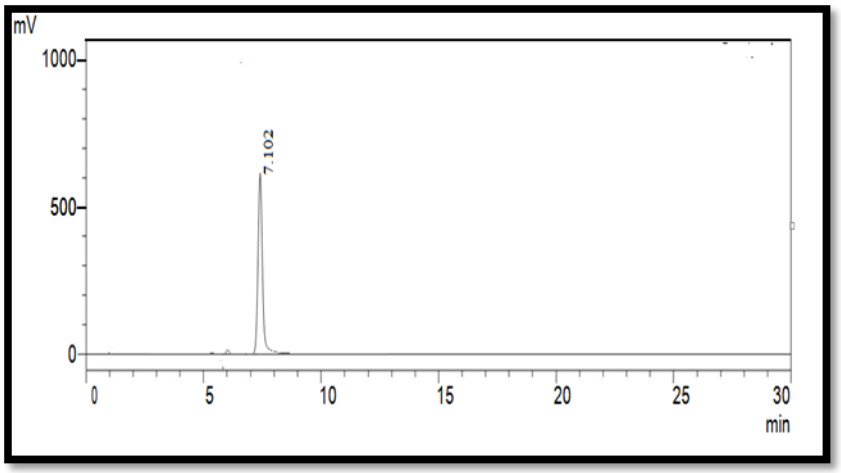


Fig. 3: Chromatogram of Standard Drug DCV (10 µg/ml).

- **Optimization of Chromatographic Condition**

The following chromatographic condition were established by trial and error and were kept constant throughout the experimentation

Stationary phase : Inertsil C18 (250 mm × 4.6ID,5 um)
Mobile phase : Methanol: Water (90:10)
pH : 03
Detection wavelength : 317 nm
Flow rate : 0.8 ml/min
Temperature : Room temperature

Run time : 30 min

Retention time : 7.10 min

The method was validated with respect to parameter like linearity, accuracy, precision, ruggedness, LOD, LOQ and repeatability.

➢ **Linearity and Range**

The linearity and range of the method was performed. From the obtained data, calibration graph were plotted using peak areas of standard drug vs. concentration for establishing linearity and range of the method shown in figure. The Daclatasvir was found to be linear in the concentration range 10 -50 ul/ml.depicted in table.

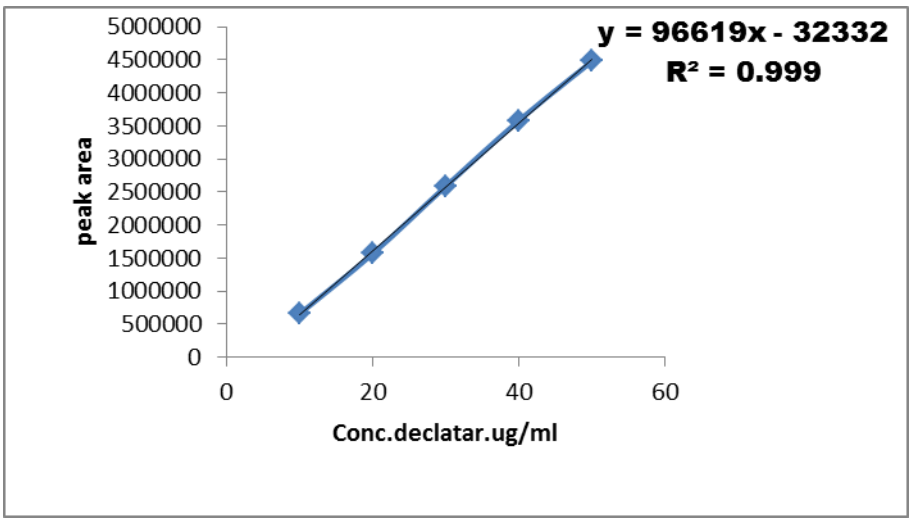


Fig. 4: Calibration Curve of DCV (10-50ul/ml) at 317 nm.

Table 5: Data for calibration curve of DCV by optimized method.

Sr. no	Conc. ug/ml	Peak area (±SD)*	%RSD
1	10	862461 ± 0.363	1.045
2	20	1641422± 1.088	0.976
3	30	2599272± 1.862	0.945
4	40	3370288± 2.181	0.876
5	50	4160086± 3.441	0.850

**(n=6) number of determination*

➢ **Accuracy**

Accurately weighed quantities of preanalysed tablet powder equivalent to 10 mg Daclatasvir was taken in 10.0 ml volumetric flask and then known amount of Daclatasvir was added at different concentration levels so as to produce solutions containing 80%, 100% and 120% of the label claim. The contents in the flasks were shaken with HPLC grade methanol volumes

were adjusted upto the mark. The solutions were filtered through a 0.45µm- membrane filter. The content of drug was calculated.

Table 6: The Result of accuracy studies.

Initial amt of formulation (ug/ml)	Standard added (ug/ml)	Area	Mean	SD	Accuracy %RSD
20	10	993652	986281	6388	0.65%
	10	982836			
	10	982356			
20	30	1005689	1020745	13045	1.28%
	30	1027862			
	30	1028683			
20	50	1002598	1019601	14734	1.44%
	50	1028635			
	50	1027569			

- **Precision**

The precision of the method was checked by carrying out repeatability, intraday and interday precision as described in section. Result of precision studies expressed in % RSD according to ICH guidelines acceptable limit (< 2) which indicates good repeatability and low variability in inter-day.

For intraday and interday study solution at single concentrations (10µg/ml) were prepared using stock solution. The absorbance of the resulting solutions was recorded at 317 nm and the obtained data were used to calculate S.D. and %R.S.D.

Table 7: Result of repeatability of measurement.

Repeatability				
Sr. no	**Conc (ug/ml)**	**Peak Area**	**Mean**	**%RSD**
1	10	962461	973318	1.17
2		957371		
3		986345		
4		976453		
5		973812		
6		983465		

**(n=6) number of determination*

Table 8: Result of interday precision.

Intraday Precision				
Sr. no	**Conc(ug/ml)**	**Peak area**	**Mean**	**%RSD***
1	10	952534	982594	1.62%
2		996253		
3		987654		
1	10	978765		
2		986824		
3		993536		

**(n=3) number of determination*

Table 9: Result of intraday precision.

Intraday precision				
Sr. no	**Conc.(ug/ml)**	**Peak area**	**Mean**	**%RSD***
1	10	1019758	1016242	1.44%
2		1006574		
3		1011017		
1	10	1010856		
2		1005025		
3		1044221		

**(n=3) number of determination*

- **LOD and LOQ**

Limit of detection (LOD) and limit of quantification (LOQ) of the development method were determined by dilution progressively low concentration of standard solution. It is calculated by using slope and standard deviation from linearity and precision respectively:

Limit of detection (LOD):

LOD = 3.3 × SD / Slope

LOQ = 10 × SD / Slope

Where, SD – Standard deviation

The LOD and LOQ were calculated as per the equation given in section. The values of LOD were found to be 0.845ug/ml for DCV. and the calculated LOQ values were found to be 0.054ug/ml. The low values of LOD and LOQ indicates the sensitivity of the method**.**

- **Degradation study**
- **Acidic hydrolytic degradation**

To 1 ml of s tock solution Daclatasvir, 1 ml of 0.1N Hcl was added into separate 10ml std flask and refluxed for 1 hour at 60 ^{0}C .The resultant solutions was diluted to obtain 100µg/ml

solution of Daclatasvir respectively with mobile phase and 10 µl solution was injected into the system and the chromatogram was recorded to assess the stability of sample.

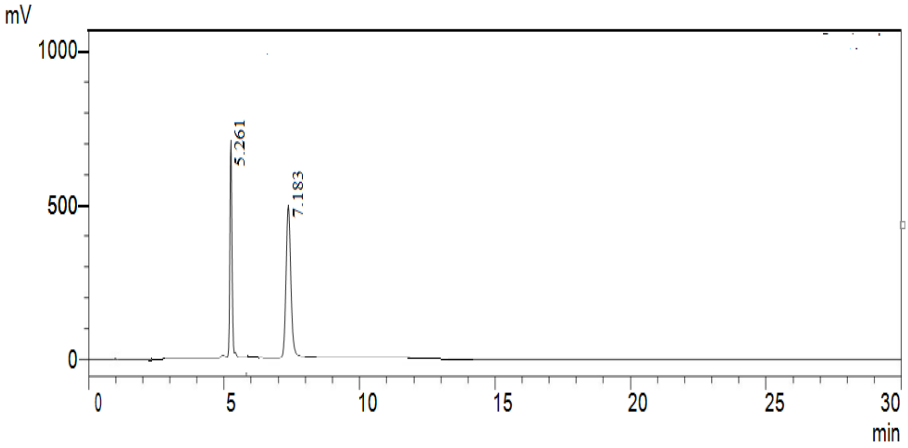


Fig 5: Chromatogram for acidic hydrolytic degradation of DCV on HPLC.

- **Basic hydrolytic degradation**

To 1 ml of s tock solution of Daclatasvir, 1 ml of 0.1M NaOH was added into separate 10ml std flask and refluxed for 1 hour at 60 ^{0}C. The resultant solutions was diluted to obtain 100µg/ml solution of Daclatasvir respectively with mobile phase and 10 µl solution was injected into the system and the chromatogram was recorded to assess the stability of sample.

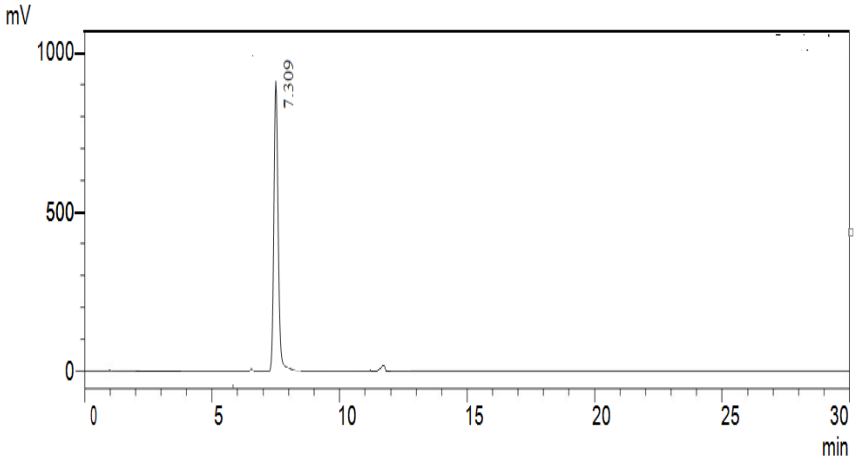


Fig. 6: Chromatogram for basic hydrolytic degradation of DCV on HPLC.

- **Oxidative degradation**

To 1 ml of s tock solution Daclatasvir, 1 ml of 30% H2O2 was added into separate 10ml std flask and refluxed for 12 hour at 60 ^{0}C. The resultant solutions was diluted to obtain 100µg/ml solution of Daclatasvir respectively with mobile phase and 10µl solution was injected into the system and the chromatogram was recorded to assess the stability of sample.

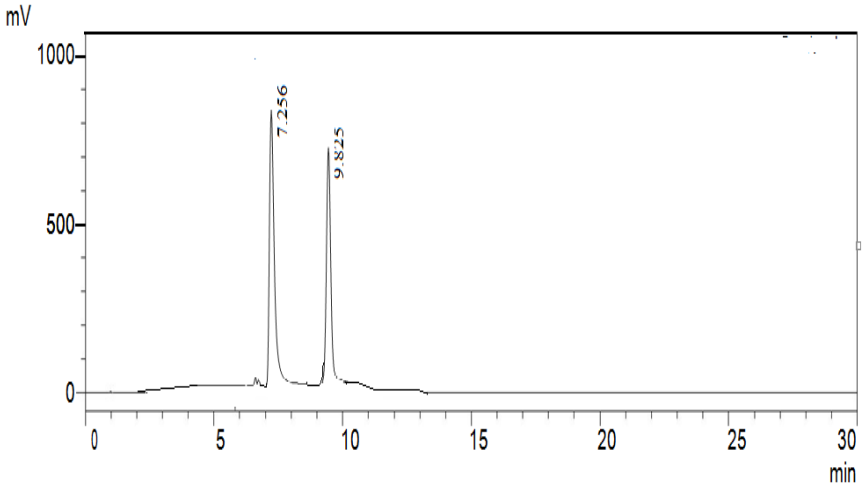


Fig. 7: Chromatogram for oxidative degradation of DCV on HPLC.

- **Thermal degradation**

To 1 ml of s tock solution Daclatasvir was added into separate 10ml std flask and refluxed for 1 hour at 80 ^{0}C. The resultant solution was diluted to obtain 100µg/ml solution of Daclatasvir respectively with mobile phase and 10µl solution was injected into the system and the chromatogram was recorded to assess the stability of sample.

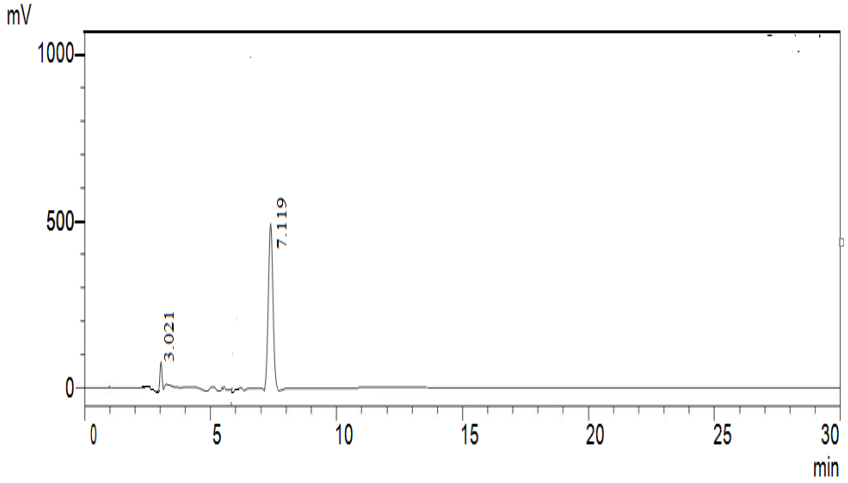


Fig. 8: Chromatogram for thermal degradation of DCV on HPLC.

- **Photolytic degradation**

1mg of Daclatasvir was placed in Petri plate and exposed to sunlight for 1 hour and later the volume was made up to mark with methanol then aliquot portion of above solution was diluted with mobile phase as a methanol (80%): water (20%) to get final concentration of about 100µg/mL and 10µL of sample solutions were injected and analyzed against control samples (lacking of degradation treatment). Daclatasvir is found to be degraded in Photolytic condition.

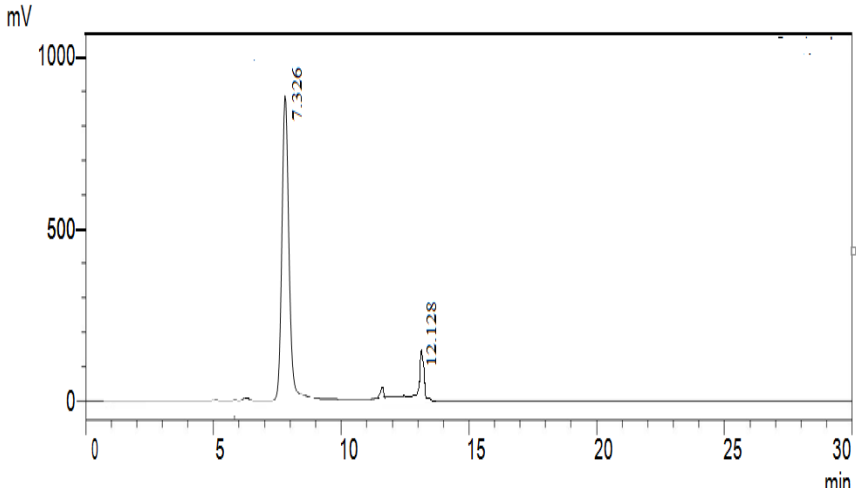


Fig. 9: Chromatogram for Photolytic Degradation of DCV on HPLC.

CONCLUSION

A validity stability indicating method was achieved HPLC. The validation of the system carried out effectively indicating method to be linear, precise, accurate, specific and robust. The stability studies on the drug were carried out successfully. The drug which when subjected to thermal, photolytic, oxidative, and acidic stress degraded into many degradation products. In most of the cases, the degradation rate was seen to be directly proportional to the amount of stress applied. The thermal stress was increased by increasing the incubation temperature, the faster the degradation took place. The more the concentration of H_2O_2 faster the drug degraded. Displayed a uniform rate of degradation when acidic stress applied. It showed a very high degradation rate when photolysis using UV radiations. The areas of degraded peaks were found to be lesser than area of standard drug concentration indicating that DCV undergo degradation under all condition.

ABBREVARTIONS

HPLC-High Performance Liquid Chromatography

ICH -International Council for Harmonization

RSD -Relative Standard Deviation

SD -Standard Deviation

Qty –Quantity

°C -Degree Celsius

Fig. –Figure

Qty -Quantity

% -Percentage

DCV- Daclatasvir

LOD - Limit of detection

LOQ - limit of quantification

ml – Milli liter

H_2O_2 –Hydrogen peroxide

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