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Development and Validation of a Stability-Indicating RP-HPLC Method for the Quantification of Daclatasvir in Tablet Formulation.

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ABSTRACT

A robust, stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantification of Daclatasvir in tablet formulations, employing separate methods for dissolution and assay determination. The dissolution method utilized an Inertsil C18 column (150 × 4.6 mm, 5 µm) with gradient elution comprising phosphate buffer (pH 2.5) and acetonitrile at a flow rate of 1.3 mL/min, with detection at 318 nm. Conversely, the assay method employed an isocratic elution using phosphate buffer (pH 7.0) and acetonitrile (60:40 v/v) on the same column at a flow rate of 1.5 mL/min. Both methods demonstrated excellent specificity, linearity ($r^2 > 0.999$), precision (%RSD < 2%), and accuracy within the acceptable recovery range of 98–102%. Forced degradation studies confirmed the methods' capability to distinguish Daclatasvir from its degradation products, affirming their stability-indicating nature. The validated methods comply with ICH Q2 (R1) guidelines and are suitable for routine quality control and stability testing of Daclatasvir in pharmaceutical formulations, ensuring reliable assessment of both dissolution profiles and assay content....

Keywords: *Daclatasvir; RP-HPLC; Dissolution; Assay; Stability-indicating method; Method Development; Method validation; Pharmaceutical analysis; Antiviral*

1. INTRODUCTION

1.1 Hepatitis C

Hepatitis C virus, or HCV, are the viruses that do not cause any visible cell damage or cell death to the infected host cells. These are called non-cytopathic viruses. NCP viruses can sometimes lead to productive infections that do not result in death of the host cells. These viruses often establish chronic infections and are better able to evade the immune response. HIV is a case in point, as are some strains of the hepatitis virus. The etiologic agent of this infection that progresses to the development of hepatitis C (the host cells might continue to proliferate and function normally, which complicates finding out and treating) is a cause of this infection which leads to the development of hepatitis C. Acute hepatitis C patients have a 60–80 percent probability of developing chronic form of the disease, when the virus has managed to override the innate and adaptive immune defences of the host [1–4]. There are seven established genotypes and 67 subtypes of HCV [5]. These genotypes have implications for the choice of antiviral drugs and are responsible for the prognostic differences in hepatitis C disease [6,7].

In this study, the awareness of HCV was found to be poor in the public in Serbia, which is inconsistent with information reported by the World Health Organization (WHO) that approximately 71 million individuals are chronically infected with HCV worldwide [8,9]. Because they are typically asymptomatic for many years and have not been tested for HCV, most individuals with chronic HCV are not aware of their HCV infection

[8,10,11]. Of 30,140 participants in the United States who completed the NHANES surveys between 2001-2008, 393 were HCV-infected at the time of the survey or had cleared the infection. Of the 170 patients who could be followed up, 49.7% knew that they were infected with the influenza virus before visiting the hospital [12]. It is estimated that only 10 to 40% of HCV-infected persons in Europe currently know that they have been infected with the virus [13,14].

In the Western world, HCV infection is the primary cause of HCC, the major complication of chronic liver disease, and the major indication for liver transplantation [7,13,15,16]. Additionally, various extra-hepatic manifestations (EHMs) have been linked with chronic HCV infection [17–21]. The first immunological response in CHCV and EHMs is a Th1 response. In addition, pathogenesis of liver injury and cirrhosis is regulated by the chemo-attraction of inflammatory infiltrates through CXCL9, -10, -11 [22, 23].

The American Association for the Study of Liver Diseases (AASLD) provides a detailed and continuously updated set of guidelines on risk factors, testing, surveillance, and evaluation for HCV infection, as well as on current treatment [7]. Prevalence of HCV testing There is considerable differences on the worldwide basis and between countries on prevalence of testing, especially in LMIC (low and middle-income countries) [24]. In line with the World Health Organization's Global Health Sector Strategy, 80% of HBV and HCV infected individuals will be treated, and 90% tested [25].

The World Health Organization (WHO) collected data and reported on the burden of disease and service provision of hepatitis B and C across all 194 Member States. This was a decision made to track and assess how far the world has come in the commitment to eradicate viral hepatitis as a public health threat. A detailed description of the methods used for global, regional and national burden of disease and service coverage data in this report can be found in this annex. This report includes data and estimates that have been reviewed and validated during consultation with the Member States in order to support them in the development of evidence-based policy framework for the elimination of viral hepatitis as a public health threat and to track the progress at country, regional and global levels towards the attainment of that target [26].

Historical Perspective

The historical perspective of Hepatitis C (HCV) is an intricate narrative of discovery, epidemiological challenges, and groundbreaking medical advancements. Before 1970s Hepatitis was recognized as a disease for centuries, with descriptions dating back to ancient civilizations. However, specific forms of viral hepatitis were indistinguishable. In the mid-20th century, many cases of hepatitis followed blood transfusions or organ transplants. These were initially attributed to hepatitis A and B, but many cases did not fit their profiles, suggesting another unidentified pathogen [27].

In 1989, scientists at Chiron Corporation, in collaboration with the CDC, identified the hepatitis C virus using recombinant DNA technology. This breakthrough was achieved by cloning parts of the viral genome, marking a milestone in virology [30,31]. By the early 1990s, HCV screening became standard in many countries, dramatically reducing transmission via transfusions and transplants. Over 58 million people worldwide are estimated to live with chronic HCV infection, with millions more unaware of their status. Efforts continue to improve access to diagnosis, treatment, and prevention, including harm-reduction strategies for people who inject drugs and broader public health campaigns [32-36].

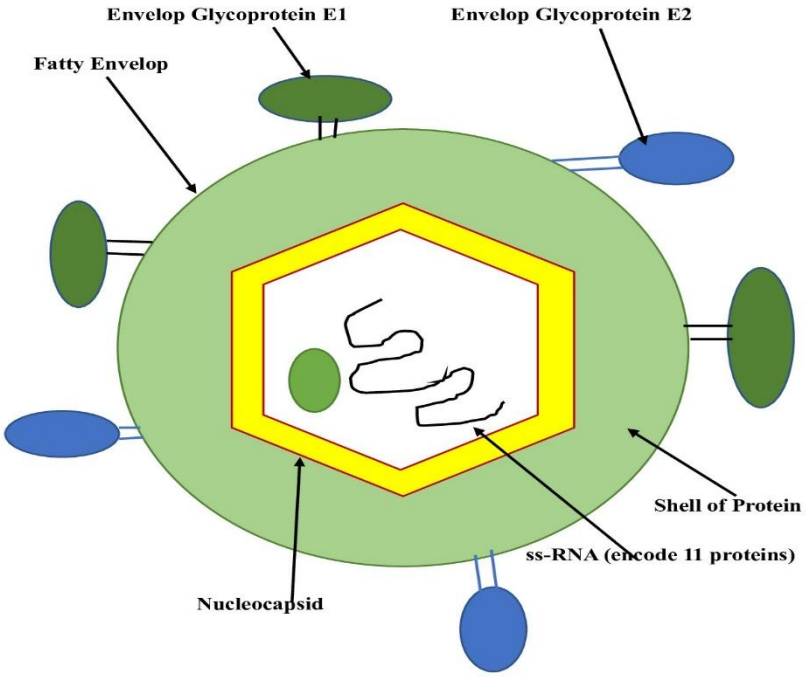

Figure 1: Structure of Hepatitis C

1.3 Chromatography

A drug is an agent administered to a recipient to diagnose, treat, manage, or prevent a disease (37). Pharmaceutical chemistry is the science of drugs and their development, that is, involved with the discovery and design of new drugs. This may involve the strategies for synthesis and their chemical structures composition, interaction with biological systems (38). To ensure the purity of drug, it is necessary to measure the content of active pharmaceutical ingredient (API) in various forms (bulk, single dose unit, and combined dose). Furthermore, determination of drug and metabolite concentrations in biological fluids after administration is also important in therapeutic drug monitoring (39). One central sub-discipline in pharmaceutical chemistry is medicinal chemistry. Instrumental analysis is a fashionable term for instrumental techniques, such as, supercritical fluid chromatography, gas or liquid (40). Chromatography, a key analytical technique, was developed by Russian botanist Mikhail Tswett in 1903. He proved separation of plant pigments by chromatography and column chromatography with different adsorbents and solvents (41). Chromatography operates on the basis of differences in solute migration between mobile and stationary phases. This method is based on the repeated adsorption–desorption cycles, leading to the separation of mixture compounds. The chromatography can be broadly categorized into two types: adsorption chromatography and partition chromatography (42).

Development of LC With the development of instrumental analysis in the late 1960s and the introduction of LC as a mature analytical method for complex samples by the late 1980s, LC underwent an impressive revival. Although the early development was rather fast, the progress of LC is much less rapid in the past few decades as it continues to impact the development of other separation technologies, including GC (43). Analytical method development is an important activity involving the establishment of the experimental conditions for the analysis of pharmaceutical compounds. This process could start with borrowing from an existing technique or developing one de novo according to scientific principles. An optimal aim is to set up a procedure that reports accurate and precise data fit for the analytical purpose (44).

1.3.1 High/Ultra-Performance Liquid Chromatography

HPLC and UHPLC are among advanced analytical methods used in the pharmaceutical, environmental, and food areas. These techniques are essential for quality control, drug analysis, and trace-level contaminant detection (45, 46). In the pharmaceutical world, HPLC/UHPLC is a critical tool used to facilitate active pharmaceutical ingredients (APIs) excipients, and finished dosage forms purity, potency, and stability testing. It is widely employed in pre-production and production stages for regular quality assurance and regulatory adherence (47). In the environmental field, HPLC/UHPLC is applied to monitor pollutants, including pesticides, herbicides, and endocrine-disrupting compounds, in water, soil, air, etc., samples (48). In food production, the methods are essential for trace amounts and monitoring of food additives, preservatives, toxins, and nutrition (49). The advantages of HPLC/UPLC are the resolution, identification, and determination of several analytes in complex matrices with good accuracy and precision. Such methods employ a detector such as UV, PDA and MS to track the analyte as it emerges from the chromatographic column (50). Routine analyses have traditionally been performed with UV or PDA detectors, which are inexpensive and simple, with mass spectrometry offering enhanced sensitivity and structural information for trace level detection and analysis of unknowns (51). UHPLC (an evolution of the HPLC technique) runs at higher pressure than traditional HPLC, due to its smaller particle size columns. This leads to better resolution, quicker analysis times and greater sensitivity, which have clear advantages in a high throughput laboratory, especially (52).

1.4 Drug Profile

Structure:

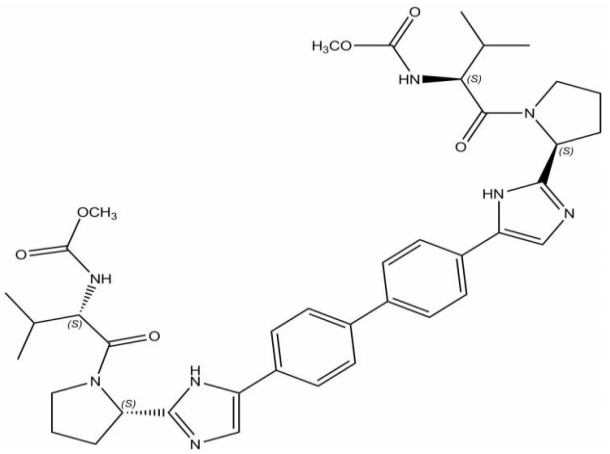

Figure 2: Structure of Daclatasvir

General profile of Daclatasvir:

Category: Direct-acting antiviral agent

Chemical Name: Dimethyl N,N'-([1,1'-biphenyl]-4,4'-diylbis{1H-imidazole-5,2-diyl-[(2S)-pyrrolidine-2,1-diyl][(2S)-3-methyl-1-oxobutane-1,2-diyl]})dicarbamate

Molecular Formula: $C_{40}H_{50}N_8O_6$

Molecular Weight: 738.89 grams per mol

pKa Value: 11.15

Solubility: Solubility of API of Daclatasvir was checked in various solvents. Thus, Methanol and HPLC water were selected for use in the mobile phase.

1.5 Validation Parameters

Analytical method validation is essential to ensure the reliability and reproducibility of results. According to International Conference on Harmonisation (ICH) guidelines (ICH Q2(R1)), the following parameters are key in method validation (53).

1.5.1 System Suitability: System suitability testing verifies that the analytical system is performing adequately before sample analysis begins. Parameters typically evaluated include retention time, peak area, resolution, theoretical plates, and tailing factor. This ensures that the chromatographic system can generate accurate and precise results under defined conditions (53, 54).

1.5.2 Specificity: Specificity is the method's ability to measure the analyte response in the presence of potential interferences such as impurities, degradants, and matrix components. A method is specific if it can unequivocally assess the analyte in a mixture without interference (53). It is particularly critical for stability-indicating methods used in pharmaceutical analysis (55).

1.5.3 Linearity and Range: Linearity refers to the ability of the method to produce results that are directly proportional to the concentration of analyte in the sample within a specified range. The range is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with suitable precision, accuracy, and linearity (53). Linearity is usually evaluated through a calibration curve with a correlation coefficient (R^2) typically greater than 0.999 (56).

1.5.4 Accuracy: Accuracy expresses the closeness of agreement between the value found and the true value. It is commonly evaluated by recovery studies, where known amounts of standard are added to the sample matrix, and the percentage recovery is calculated (53). Acceptable recovery ranges typically lie between 98-102% depending on the concentration (57).

1.5.5 Method Precision: Precision, or repeatability, assesses the consistency of the method under the same operating conditions over a short interval of time. It is usually expressed as the relative standard deviation (RSD) of replicate measurements. A low RSD (e.g., <2%) indicates good repeatability (53, 58).

1.5.6 Solution Stability: Stability testing determines whether the analyte remains stable in the prepared solution over a specific period under defined storage conditions. This ensures that degradation does not affect analytical results during the analysis window (59). Stability is typically evaluated by comparing results at initial and later time points.

1.5.7 Robustness: Robustness tests the method's capacity to remain unaffected by small deliberate changes in method parameters like pH, temperature, or flow rate. It ensures method reliability under routine usage. Slight variations should not significantly impact results, indicating a robust method (53, 60).

1.5.8 Filter Compatibility: Filter compatibility ensures that the filtration process does not lead to significant loss of the analyte or introduce contaminants. The method's performance is compared between filtered and unfiltered samples or between different types of filters to ensure consistency in results (61).

2. Materials and Methods

2.1 Selection of Mobile Phase

Daclatasvir was dissolved in common laboratory solvents like water, acetonitrile (ACN), methanol (MeOH), abs. ethanol, a mixed ratio of water & acetonitrile, ortho phosphoric acid, a mixed ratio of water & methanol, etc. Acetonitrile and HPLC water were selected in the use in mobile phase after tests because of the best drug solubility, easy availability and low cost.

2.2 Preparation of Mobile Phase

2.2.1 Mobile Phase A. a buffer solution prepared by dissolving 1.36 g of potassium dihydrogen orthophosphate in 900 ml of water, add 2 ml of triethylamine, mix and dilute to 1000 ml with water, adjusted to pH 2.5 with dilute orthophosphoric acid.

2.2.2 Mobile Phase B. Acetonitrile.

2.3 Preparation of diluent

A combination of HPLC Grade Milli Q Water & Methanol in the proportion (50:50) was prepared and utilized as the solvent mixture/diluent.

2.4 Preparation of standard stock solution

A 2400 PPM solution of Daclatasvir API was prepared and used as the standard stock solution. About 60.0 mg drug was weighed accurately and transferred into a 25 ml volumetric flask and then 5.0 ml of methanol was added into it, and the

volumetric flask was shaken for intermittent mixing and sonicated for 5 minutes. Finally, made up its volume up to mark with methanol.

2.5 Preparation of Standard Solution

5.0 ml of stock solution was taken in 200 ml volumetric flask. Around 20 ml of solvent mixture was added into it and degassed using sonicator for a few minutes. Finally, made up to volume up to 200 ml mark with solvent mixture.

2.6 Preparation of Test Solution

1 Tablet was dispersed into 1000 ml of dissolution medium 50:50 (methanol:water) at 75 rpm for 30 minutes.

2.7 Selection of Wavelength for UV Detection

Solution of Daclatasvir was prepared in solvent mixture. UV spectrum was obtained by scanning solution over the entire range (200-400). Daclatasvir showed maxima at 318 nm and was selected for the method.

2.8 HPLC Method Development and Optimisation

List of columns that were tested in our development process:

Inertsil [C-8] (150×4.6 mm, 5 um)

Waters [C-8] (50×4.6 mm, 5 um)

Hypersil [BDS C-8] (150×4.6 mm, 3.5 um)

Thermo [C-8] (150×4.6 mm, 5 um)

It was found that ThermoScientific C-18 (150 mm length × 4.6 mm width, 5 um pore size) column gave the best result and the temperature of the column oven was then fixed at 35ºC.

Different flow rates like- 1.0 ml per min, 1.20 ml per min, 0.80 ml per min, etc. were also tested. However, the best results for the retention time, peak, no. of plates, tailing factor, etc. were only achieved at the flow rate 1.3 ml per min flow rate and the final HPLC/UPLC test method was optimized using these conditions only.

2.9 Chromatographic conditions

Table 1: Final chromatographic conditions used to validate the proposed HPLC method for Dissolution

1.	**HPLC System Used**	Thermo Scientic Dionex 3000
2.	**Software used**	Chromeleon 7
3.	**Column**	Inertsil C18 (150×4.6 mm, 5 um)
4.	**Column Temperature (°C)**	35
5.	**Sample Temperature (°C)**	10
6.	**Flow Rate (ml/min)**	1.3
7.	**Injection Volume (microliters)**	10.0
8.	**Wavelength (nm)**	318
9.	**Run Time (mins)**	30 min
10.	**Elution Mode**	Gradient
11.	**RPM (Rotation Per Minute)**	30 minutes

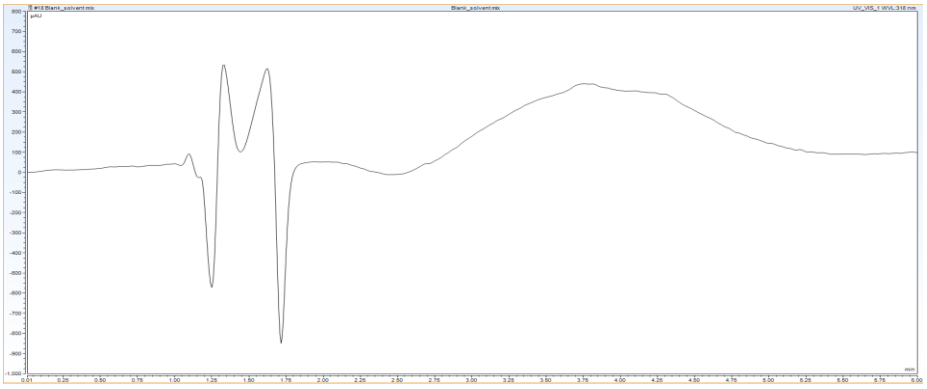

Figure 3: Chromatogram for Blank

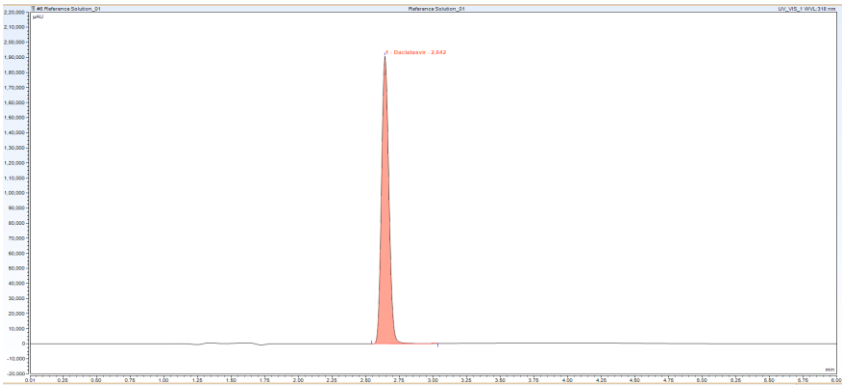

Figure 4: Chromatogram for standard Daclatasvir

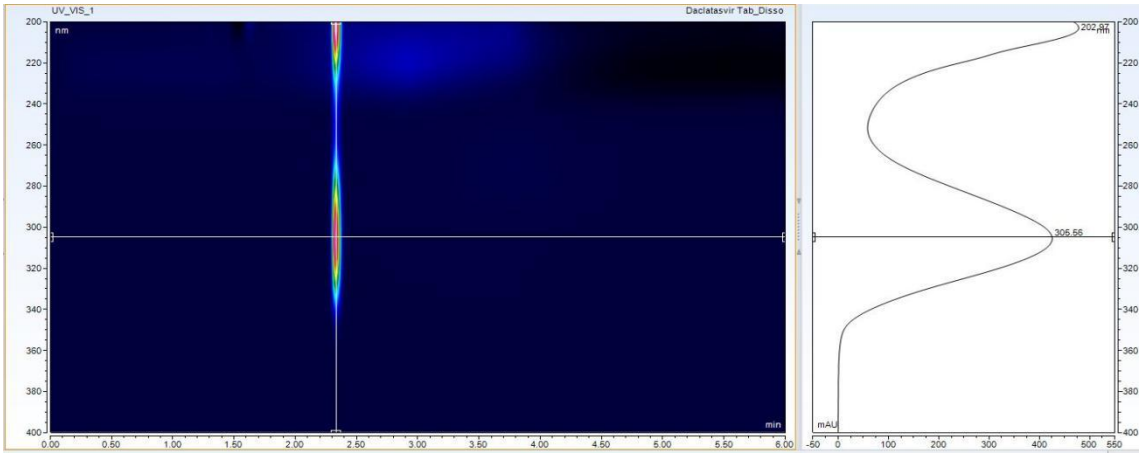

Figure 5: Chromatogram for Peak Purity of Daclatasvir

2.10 Validation Parameters

2.10.1 Specificity

The test method passes the test no interference observed at analyte's retention time or detection wavelength. the peak purity found to be 950.

2.10.2 Accuracy

Accuracy was carried out at different three levels at 50%, 100% and 150% in triplicate and % Recovery within acceptable range (e.g. 98%–102%). Result % recovery within acceptable range (e.g. 98%–102%). Results are described in Table 2 given below

Table 2: Result of Accuracy of Daclatasvir (50 - 150 % Concentration)

S. No.	Accuracy Levels	Accuracy (%)	Mean	SD	RSD (%)
1.	At 50% Level -01	100.34	100.19	0.22	0.22
2.	At 50% Level -02	100.03			
3.	At 100% Level -01	99.03	99.59	0.79	0.79
4.	At 100% Level -02	100.15			
5.	At 150% Level -01	100.08	100.69	0.86	0.85
6.	At 150% Level -02	101.29			
Overall Mean			**100.15**	**0.72**	**0.72**

The test method passes the test, and RSD was found to be less than 2.0 %.

2.10.3 Precision

A solution of 60 PPM (ug/ml) solution for Daclatasvir was prepared and Method Precision tests were carried out. Procedure allowed for Intraday precision and Inter-day precision are given. The analytical test method is precise when the values of a multiple sampled series of the same analyte are very close to each other. Results are stated in Table 4 and 5 respectively given below.

Repeatability: 6 Reps of Daclatasvir was performed and %RSD was calculated as follows:

Table 3: Result of Repeatability Study of Daclatasvir (60 PPM)

S. No	Area	% Assay
1	737485	90.80
2	747024	91.98
3	730977	90.00
4	756080	93.09
5	746702	91.94
6	740868	91.22
AVG	743189	91.51
STD	8721	1.07

%RSD	1.17	1.17

The test method passes the test as the RSD was less than 2%.

Intermediate Precision

DAY-1: 6 Reps of Daclatasvir was performed for inter-day precision and %RSD was calculated as follows:

Table 4: Result of Intermediate Precision (Inter Day-1) of Daclatasvir (60 PPM)

S. No	**Area**	**% Assay**
1	737423	90.79
2	731197	90.03
3	725316	89.30
4	728797	89.73
5	730927	89.99
6	746783	91.95
AVG	733407	90.30
STD	7652	0.94
%RSD	**1.04**	1.04

The test method passes the test as the RSD was less than 2%.

2.10.4 Filter Compatibility:

Filter compatibility was evaluated for both reference and test solutions to ensure that filtration does not affect analyte recovery. For the validated method, all results were found within acceptable limits stated below in Table 6

Table 5: Filter Compatibility Results for Reference Solution

S. No	**Filter**	**Average Area**	**Absolute % Area Difference**
1.	As such/ Centrifuge	718000	0.00
2.	PVDF	718115	0.02
3.	NYLON	725226	1.01

Table 6: Filter Compatibility Results for Sample Solution

S. No	**Filter**	**Average Area**	**Absolute % Area Difference**
1.	As such/ Centrifuge	714628	0.00
2.	PVDF	716337	0.24

3.	NYLON	722730	1.13

The test method passes the test as the Percent (%) Area Difference was less than 2%. **Solution**

2.10.5 Solution Stability:

Stability of analyte in the sample solution over time under set conditions. No significant degradation results remain within acceptable limits over the tested period. Results are described under Table 8.

Table 7:Results of Reference Solution and Sample Solution for Solution Stability

S. No.	Time	Area of Reference solution	Cumulative % RSD	Time	Area of Sample Solution	Cumulative % RSD
1.	Initial	717787	0.00	Initial	714841	0.00
2.	After 02 hrs 44 mins	716848	0.09	After 02 hrs 15 mins	719888	0.50
3.	After 06 hrs 45 mins	720005	0.23	After 06 hrs 16 mins	710250	0.67
4.	After 10 hrs 47 mins	717002	0.20	After 10 hrs 18 mins	712830	0.57
5.	After 14 hrs 48 mins	723573	0.39	After 14 hrs 19 mins	713256	0.50
6.	After 18 hrs 50 mins	722231	0.40	After 18 hrs 21 mins	711067	0.48
7.	After 21 hrs 05 mins	719558	0.36	After 20 hrs 36 mins	711559	0.46
8.	After 25 hrs 06 mins	722620	0.37	After 24 hrs 38 mins	714871	0.43
9.	After 27 hrs 00 mins	720260	0.34	After 26 hrs 31 mins	712059	0.41
10.	After 31 hrs 02 mins	723186	0.35	After 30 hrs 33 mins	714841	0.39
11.	After 35 hrs 03 mins	726354	0.42	After 34 hrs 34 mins	713589	0.37
12.	After 39 hrs 05 mins	725874	0.45	After 38 hrs 36 mins	709658	0.39
13.	After 43 hrs 06 mins	728454	0.51	After 42 hrs 37 mins	694444	0.82

Result – The Drug is stable till 42 Hours 37 minutes in solution stability test.

Force Degradation

The Daclatasvir sample was found to be within the specified limits for degradation under the conditions of the forced degradation test, indicating that the drug maintained its stability and potency even when exposed to a variety of stress factors, such as heat, humidity, and acidic or alkaline conditions. These results suggest that Daclatasvir can withstand these conditions without significant loss of efficacy, ensuring its reliability and safety throughout its shelf life. The % degradation of Daclatasvir was shown in table 8.

Table 8: Results of Forced Degradation Under Various Conditions

S.No.	Stress Conditions	% Degradation
1.	Treated with 5N HC1 solution at 60°C temperature for about 4 hours.	0.307
2.	Treated with 5N NaOH solution at 60°C temperature for about 4 hours.	0.213
3.	Treated with 30% H202 solution for about 4 hours	0.522
4.	Treated with water at 60°C temperature for about 4 hours.	0.449
5.	Exposed to humidity at 25°C/90% RH for about 48 hours.	0.275
6.	Exposed to heat at 105°C temperature for about 72 hours.	0.679

3.1 Robustness

The Daclatasvir sample was found to be within the specified limits under various conditions tested for robustness, including variations in flow rate, temperature, pH, and changes in mobile phase concentration. These factors were systematically altered to assess the drug's stability and performance under different analytical conditions. The results demonstrated that Daclatasvir maintained its integrity and fell within the acceptable limits for each of these parameters are stated in Table 9, indicating the method's reliability and the drug's resilience to typical variations encountered during the testing process.

Table 9A: Results of Effect of variation in Flow Rate

System Suitability Parameters	Observed Value with flow rate			Acceptance Criteria
	1.2 ml/minute	1.40 ml/minute	1.50 ml/minute	
The tailing factor for Daclatasvir peak	1.2	0.8	1.4	NMT 2.0
The ratio of peak area of Daclatasvir from duplicate injections	1.01	0.98	1.05	Between 0.90 and 1.10

Table 9B: Results of Effect of variation in Column Oven Temperature

System Suitability Parameters	Observed Value with column oven temperature			Acceptance Criteria
	30°C	35°C	40°C	
The tailing factor for Daclatasvir peak	1.1	1.0	1.0	NMT 2.0
The ratio of peak area of Daclatasvir from duplicate injections	1.06	1.00	0.97	Between 0.90 and 1.10

Table 9C: Results of Effect of variation in pH of buffer in Mobile Phase

System Suitability Parameters	Observed Value with column oven temperature			Acceptance Criteria
	pH 2.3	pH 2.5	pH 2.7	
The tailing factor for Daclatasvir peak	0.9	0.9	0.9	NMT 2.0
The ratio of peak area of Daclatasvir from duplicate injections	0.99	1.00	1.00	Between 0.90 and 1.10

Table 9D: Results of Effect of variation in Mobile Phase composition

System Suitability Parameters	Observed Value with column oven temperature			Acceptance Criteria
	Decrease in volume of Acetonitrile	As per method	Increase in volume of Acetonitrile	
The tailing factor for Daclatasvir peak	0.9	0.9	0.9	NMT 2.0
The ratio of peak area of Daclatasvir from duplicate injections	1.00	0.99	1.01	Between 0.90 and 1.10

2. RESULTS

Selection of mobile phase

Daclatasvir was dissolved in common laboratory solvents like water, acetonitrile (ACN), methanol (MeOH), abs. ethanol, a mixed ratio of water & acetonitrile, ortho phosphoric acid, a mixed ratio of water & methanol, etc. Acetonitrile and HPLC water were selected in the use in mobile phase after tests because of the best drug solubility, easy availability and low cost.

Preparation of Mobile Phase

A mixture of 60 volumes of buffer solution prepared by dissolving 1.36 g of potassium dihydrogen orthophosphate in 900 ml of water, add 1 ml of triethylamine, mix and dilute to 1000 ml with water, adjusted to pH 7.0 with orthophosphoric acid and 40 volumes of acetonitrile.

Preparation of diluent /solvent mixture

A combination of HPLC Grade Milli Q Water & Methanol in the proportion (50:50) was prepared and utilized as the solvent mixture/diluent.

Preparation of standard stock solution

A 1080 PPM solution of Daclatasvir API was prepared and used as the standard stock solution. Accurately weighed & transferred 27.32mg of daclatasvir dihydrochloride working standard into a 25ml volumetric flask, added 10ml methanol, sonicated to dissolved, then made up to 25ml with methanol.

Preparation of standard solution

Accurately pipetted and transferred 5.0ml of this solution into a 100ml volumetric flask then made up to 100ml with solvent mixture. Filtered through 0.45µm Nylon filter.

Preparation of test solutions

Weigh and powder 20 tablets. Disperse a quantity of the powder containing 90 mg of Daclatasvir in 6 ml of water with the aid of ultrasound, add 200ml of methanol. Further sonicate for 60 minutes with intermittent shaking and dilute to 250.0 ml with methanol. Centrifuge a portion of solution at 5000 rpm for 10 minutes. Dilute 3.0 ml of the supernatant liquid to 20.0 ml with the solvent mixture.

Selection of Wavelength for UV Detection

Solution of Daclatasvir was prepared in solvent mixture. UV spectrum was obtained by scanning solution over the entire range (200-400). Daclatasvir showed maxima at 318 nm and was selected for the method.

HPLC Method Development and Optimisation

List of columns that were tested in our development process:

Inertsil [C-8] (150×4.6 mm, 5 um)

Waters [C-8] (50×4.6 mm, 5 um)

Hypersil [BDS C-8] (150×4.6 mm, 3.5 um)

Thermo [C-8] (150×4.6 mm, 5 um)

It was found that Inertsil C-18 (150 mm length × 4.6 mm width, 5 um pore size) column gave the best result, and the temperature of the column oven was then fixed at 35ºC.

Different flow rates like- 1.0 ml per min, 1.20 ml per min, 0.80 ml per min, etc. were also tested. However, the best results for the retention time, peak, no. of plates, tailing factor, etc. were only achieved at the flow rate 1.3 ml per min flow rate and the final HPLC/UPLC test method was optimized using these conditions only.

Chromatographic conditions

Table 10: Final chromatographic conditions used to validate the proposed HPLC method.

1.	**HPLC System Used**	Thermo Scientic Dionex 3000
2.	**Software used**	Chromeleon 7
3.	**Column**	Inertsil C18 (150×4.6 mm, 5 um)
4.	**Column Temperature (°C)**	30
5.	**Sample Temperature (°C)**	10
6.	**Flow Rate (ml/min)**	1.5
7.	**Injection Volume (microlitres)**	10.0
8.	**Wavelength (nm)**	318
10.	**Elution Mode**	Isocratic

Specificity

As a result, it was observed that there was no interference between the blank and placebo samples with respect to the analyte peak. Additionally, the peak purity was found to be 950, indicating that the analyte peak was pure and free from any impurities or interferences.

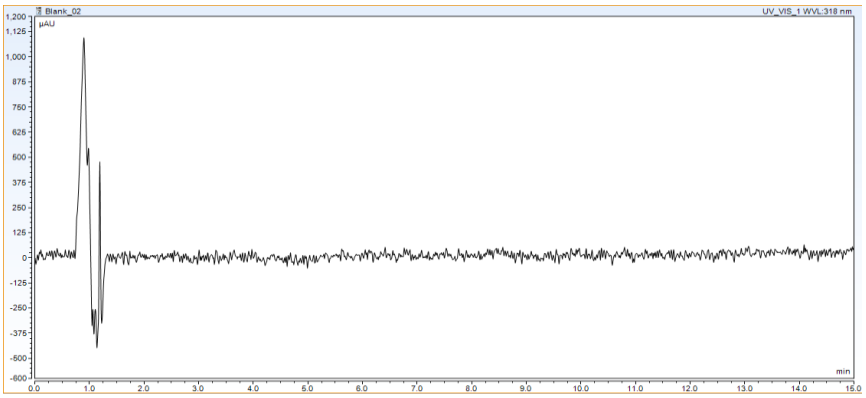

Figure 6: Chromatogram for Blank

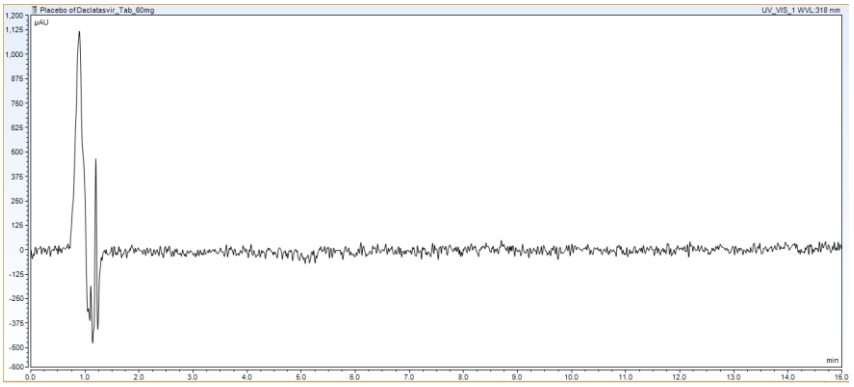

Figure 7: Chromatogram for Placebo

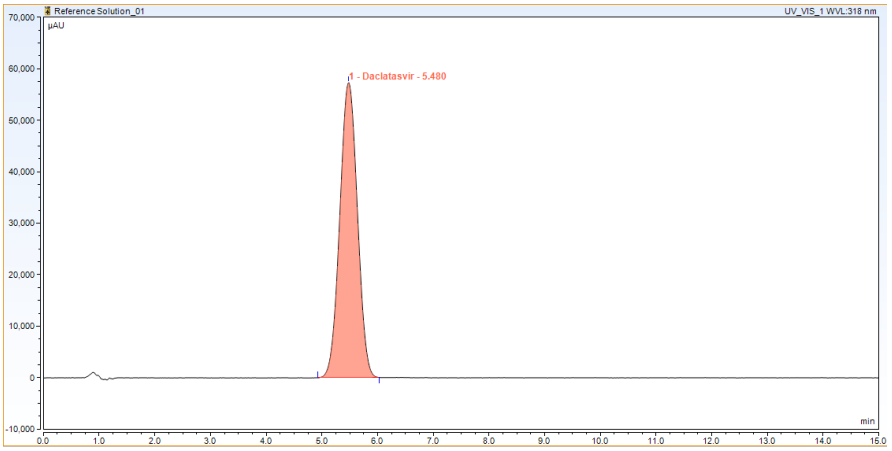

Figure 8: Chromatogram for Reference Solution

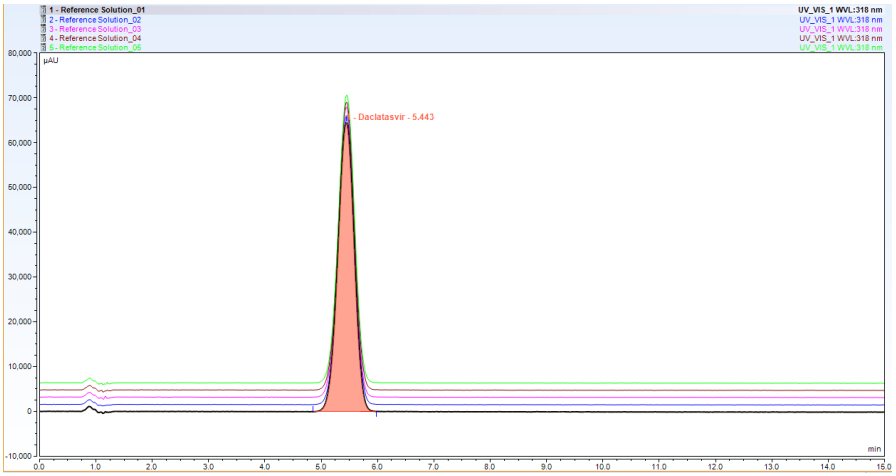

Figure 9: Chromatogram for Reference Solution (Overlay)

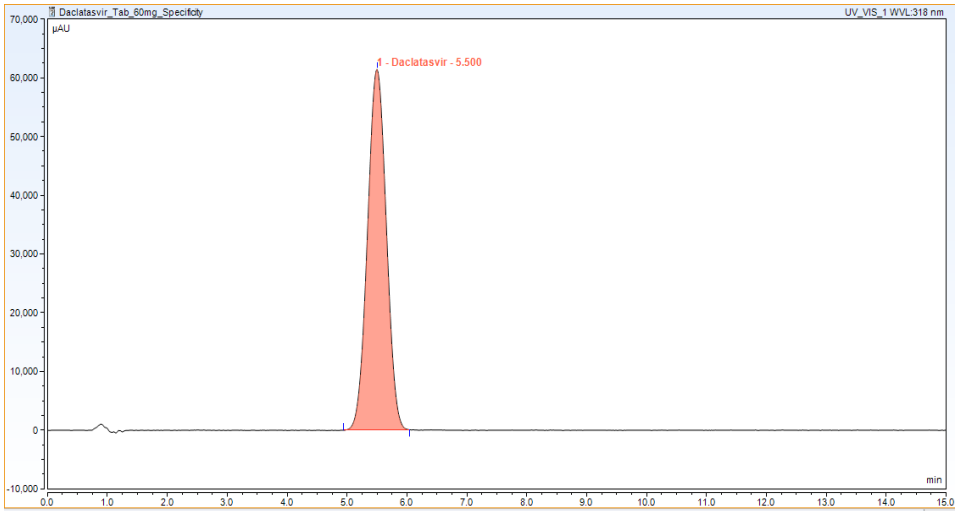

Figure 10: Chromatogram for Test Solution

Method Precision

The developed HPLC technique was assessed for research precision in accordance with ICH regulations. Six replicates of Daclatasvir samples were used to obtain the percent RSD and intermediate precision (intraday precision). For the

repeatability examination, Daclatasvir inter-day precision was examined using three different days and six Daclatasvir replicates. Results are shown below in Table 11

Table 11: Method Precision Results

S. No	Area	% Assay
1	1361214	100.54
2	1360304	100.28
3	1374490	101.33
4	1374392	101.32
5	1381114	101.63
6	1331897	98.19
AVG	1363902	100.55
STD	17680	1.27
%RSD	1.30	1.26

Linearity and range

The linearity of the analytical method was investigated by injecting five distinct level concentrations of pure Daclatasvir, ranging at 50%, 75%, 100%, 125% and150%. The slope, Y-intercepts, and correlation coefficient of each Daclatasvir concentration were obtained by plotting peak area against concentration. Peak area and concentration showed a link in the data produced by linearity studies. Regression coefficients of 0.9998 indicated that the daclatasvir technique produced linear concentration ranges. The Daclatasvir linearity curve is shown in Figure 11.

Table 12: Dilutions of Daclatasvir to evaluate its linearity across a range of concentrations

Concentrations	Stock Solution		2nd Dilution	
	Mg	Ml	ml	ml
50%	27.18	25	0.250	10
75%			0.375	10
100%			0.500	10
125%			0.625	10
150%			0.750	10

Table 13: The results of the linearity test are shown across a range of concentrations

S. No	Linearity Levels	Calculated Conc. (%)	Average Area
1.	50% Level	49.81	652589
2.	75% Level	74.47	977508
3.	100% Level	100.91	1326006
4.	125% Level	125.55	1650601
5.	150% Level	149.27	1963201
		Squared Correlation Coefficient	0.9998
		Intercept	-3746

	Slope	13177

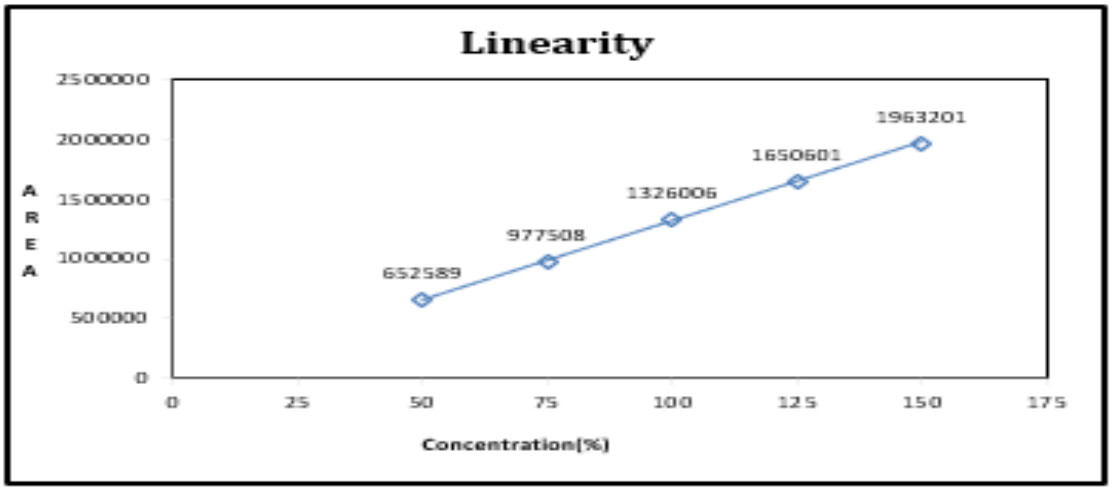

Figure 11: Linearity Curve of Daclatasvir

Accuracy

The consistency between true and experimental values establishes whether an analytical technique is correct. Three concentrations—50%, 100%, and 150% from different Daclatasvir standard curve ranges were selected to determine the accuracy. The recovery of daclatasvir was tested in duplicate for drug sample concentrations of 50%, 100%, and 150%. The effects of excipients, which are commonly employed in pharmaceutical formulations of medications, were investigated through recovery trials. The recovery of Daclatasvir in the sample showed a high degree of quantitative proficiency. The accuracy results are shown in Table 14.

Table 14: Results of Accuracy Evaluation

S. No.	**Accuracy Levels**	**Sample (mg)**		**Dilution (ml)**			**% Accuracy**	**Mean % Accuracy**	**SD**	**% RSD**
		Placebo	**API**							
1.	At 50% Level-1	383.68	45.55	250	3	20	100.37	99.91	0.40	0.40
2.	At 50% Level-2	382.96	45.84	250	3	20	99.73			
3.	At 50% Level-3	382.48	45.81	250	3	20	99.64			
4.	At 100% Level-1	383.06	91.63	250	3	20	98.74	99.28	0.65	0.65
5.	At 100% Level-2	382.64	91.20	250	3	20	100.00			
6.	At 100% Level-3	382.54	91.29	250	3	20	99.10			
7.	At 150% Level-1	383.39	138.56	250	3	20	99.86	100.56	0.62	0.62

8.	At 150% Level-2	382.80	136.13	250	3	20	101.03			
9.	At 150% Level-3	382.61	136.43	250	3	20	100.79			
								99.92	**0.74**	**0.74**

Filter Compatibility

To verify the accuracy of the method for quantifying Daclatasvir in filter compatibility, samples were centrifuged and filtered through 0.45 µm PVDF and nylon filter. Filter compatibility was computed using the peak area and it was considered that both the filters are suitable for Daclatasvir. Results of filter compatibility for both reference solution and sample solution are stated below in table 15 and 16.

Table 15: Filter Compatibility Results for Reference Solution

S. No	Filter	Average Area	Absolute % Area Difference
1.	As such/ Centrifuge	1272372	0.00
2.	PVDF	1254663	-1.39
3.	NYLON	1252793	-1.54

Table 16: Filter Compatibility Results for Sample Solution

S. No	Filter	Average Area	Absolute % Area Difference
1.	As such/ Centrifuge	1374915	0.00
2.	PVDF	1378445	0.26
3.	NYLON	1361485	-0.98

Solution Stability

Solution Stability is an essential part of method validation to ensure that prepared solutions (e.g., standard solutions and sample solutions) remain stable over the intended duration of analysis. Results are observed that cumulative RSD for reference solution was found within 2% (at 25°C) up to 52 Hrs & 50 minutes and cumulative RSD for sample solution was found within 2% (at 25°C) up to 52 Hrs & 03 minutes stated below in table 17.

Table 17 - Results of Reference Solution and Sample Solution for Solution Stability

S.No	Time	Area of Reference solution	Cumulative % RSD	Time	Area of Sample Solution	Cumulative % RSD
1.	Initial	1254158	0.00	Initial	1334778	0.00
2.	After 05 hrs 23 mins	1261196	0.40	After 04 hrs 36 mins	1343322	0.45
3.	After 09 hrs 42 mins	1248641	0.50	After 08 hrs 54 mins	1328141	0.57
4.	After 14 hrs 01 mins	1247463	0.50	After 13 hrs 13 mins	1331645	0.49
5.	After 18 hrs 20 mins	1252567	0.43	After 17 hrs 32mins	1335662	0.42

6.	After 22 hrs 38 mins	1258175	0.43	After 21hrs 34 mins	1314943	0.72
7.	After 26 hrs 57 mins	1255266	0.39	After 26 hrs 10 mins	1313094	0.84
8.	After 31 hrs 16 mins	1250755	0.37	After 30 hrs 28 mins	1309836	0.93
9.	After 35 hrs 35 mins	1247720	0.38	After 34 hrs 47 mins	1308805	0.97
10.	After 39 hrs 54 mins	1248446	0.38	After 39 hrs 06 mins	1345080	1.04
11.	After 44 hrs 13 mins	1253226	0.36	After 43 hrs 25 mins	1344194	1.06
12.	After 48 hrs 32 mins	1255559	0.35	After 47 hrs 44 mins	1339203	1.04
13.	After 52 hrs 50 mins	1253070	0.33	After 52 hrs 03 mins	1340890	1.03

Force Degradation

The Daclatasvir sample was found to be within the specified limits for degradation under the conditions of the forced degradation test, indicating that the drug maintained its stability and potency even when exposed to a variety of stress factors, such as heat, humidity, and acidic or alkaline conditions. These results suggest that Daclatasvir can withstand these conditions without significant loss of efficacy, ensuring its reliability and safety throughout its shelf life. The % degradation of Daclatasvir was shown in table 18.

Table 18: Results of Forced Degradation Under Various Conditions

S. No.	Stress Conditions	% Degradation
1.	Treated with 5N HC1 solution at 60°C temperature for about 4 hours.	0.204
2.	Treated with 5N NaOH solution at 60°C temperature for about 4 hours.	0.485
3.	Treated with 30% H202 solution for about 4 hours	0.985
4.	Treated with water at 60°C temperature for about 4 hours.	0.216
5.	Exposed to humidity at 25°C/90% RH for about 48 hours.	0.179
6.	Exposed to heat at 105°C temperature for about 72 hours.	0.322

Robustness

The Daclatasvir sample was found to be within the specified limits under various conditions tested for robustness, including variations in flow rate, temperature, pH, and changes in mobile phase concentration. These factors were systematically altered to assess the drug's stability and performance under different analytical conditions. The results demonstrated that Daclatasvir maintained its integrity and fell within the acceptable limits for each of these parameters are stated in table 19, indicating the method's reliability and the drug's resilience to typical variations encountered during the testing process

Table 19A: Results of Effect of variation in Flow Rate

System Suitability Parameters	Observed Value with flow rate			Acceptance Criteria
	1.08 ml/minute	1.20 ml/minute	1.32 ml/minute	
The tailing factor for Daclatasvir peak	1.0	0.9	0.9	NMT 2.0

The ratio of peak area of Daclatasvir from duplicate injections	1.04	1.02	0.98	Between 0.90 and 1.10

Table 19B: Results of Effect of variation in Column Oven Temperature

System Suitability Parameters	Observed Value with column oven temperature			Acceptance Criteria
	30°C	35°C	40°C	
The tailing factor for Daclatasvir peak	1.1	1.0	1.0	NMT 2.0
The ratio of peak area of Daclatasvir from duplicate injections	1.06	1.00	0.97	Between 0.90 and 1.10

Table 19C: Results of Effect of variation in pH of buffer in Mobile Phase

System Suitability Parameters	Observed Value with column oven temperature			Acceptance Criteria
	pH 2.3	pH 2.5	pH 2.7	
The tailing factor for Daclatasvir peak	0.9	0.9	0.9	NMT 2.0
The ratio of peak area of Daclatasvir from duplicate injections	0.99	1.00	1.00	Between 0.90 and 1.10

Table 19D: Results of Effect of variation in Mobile Phase composition

System Suitability Parameters	Observed Value with column oven temperature			Acceptance Criteria
	Decrease in volume of Acetonitrile	As per method	Increase in volume of Acetonitrile	
The tailing factor for Daclatasvir peak	0.9	0.9	0.9	NMT 2.0
The ratio of peak area of Daclatasvir from duplicate injections	1.00	0.99	1.01	Between 0.90 and 1.10

3. CONCLUSION

Distinct RP-HPLC methods were developed and validated for dissolution and assay determination of Daclatasvir in tablet formulations. Both methods exhibited high specificity, precision, accuracy, and robustness, fulfilling ICH validation criteria. The methods proved stability-indicating by effectively resolving Daclatasvir from its degradation products under stress conditions. These validated methods are suitable for routine quality control and stability testing of Daclatasvir tablets, ensuring reliable evaluation of both dissolution profiles and assay content.

4. FUNDING

Not applicable.

5. AUTHORS CONTRIBUTION

Himanshu. Writing – Original Draft, Data Curation, Formal Analysis and Validation, Suryakant Verma. Methodology, Jaishiv Chauhan. Investigation, Writing, Reviewing and Editing, Kshitiz Sahu and Sameeksha. Resources, Visualization and Assistance in Literature Survey, Anup Kumar Mamgain. Supervision and Final Evaluation of the Draft.

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