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A Facile RP-HPLC Method for Quantification of Antiviral Drug Daclatasvir Dihydrochloride in Both Tablet Dosage Form and River Nile Samples

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ABSTRACT

A rapid and sensitive RP-HPLC method was developed for the quantitative analysis of daclatasvir dihydrochloride in tablet dosage forms. The method utilizes a Zorbax Eclipse Plus C18 column and a mobile phase consisting of 0.02M KH_2PO_4 buffer **(**pH 3.0**)** and acetonitrile **(**70:30% v/v**)**. The method demonstrated excellent linearity **(**$R^2 > 0.9996$**)** over the concentration range of 0.15- 90µg/ mL, with low limits of detection **(**LOD**)** and quantification **(**LOQ**)**. The method was also accurate and precise, with recovery values ranging from 98.72 to 101.30% and relative standard deviations less than 2.0%. In conclusion, this validated RP-HPLC method provides a reliable and efficient tool for the routine analysis of daclatasvir dihydrochloride in tablet formulations and the Nile River samples. This method can be applied for various quality control purposes, including dissolution testing and biowaiver studies.

INTRODUCTION

Hepatitis C virus **(**HCV**)** infection is a significant public health concern, with approximately 170–180 million infected individuals worldwide **(Shepard *et al.*, 2005; Lavanchy, 2011)**. Patients infected with HCV are at risk of life-threatening complications which can lead to cirrhosis, decompensated liver disease (liver failure), hepatocellular carcinoma, and the need for liver transplantation **(Jacobson *et al.*, 2010)**. Daclatasvir dihydrochloride (Fig. 1) is a new helpful drug for treating hepatitis C genotype 3. It was developed by Bristol-Myers Squibb and was approved in Europe on 22 August 2014 and gained its FDA approval on July 24, 2015, in the United States **(US FDA Press announcements, 2015)**.

Daclatasvir is an inhibitor of HCV nonstructural protein 5A (NS5A). HCV NS5A is a multifunctional protein with key roles in HCV replication, virus assembly, and the modulation of cellular signaling pathways. DCV inhibits virion assembly as well as viral RNA replication. The IUPAC, is the name for Daclatasvir dihydrochloride. The chemical

name for it is "methyl((1S)-1-(((2S)-2-(5-(4'-(2-((2S)-1-((2S)-2-((methoxycarbonyl)amino)-3-methyl butanoyl)-2-pyrrolidinyl)-1H-imidazole-5-yl)-4-biphenylyl)-1H-imidazole-2-yl)-1-pyrrolidinyl)carbonyl)-2-methyl propyl)carbamate dihydrochloride" with the CAS number [1009119-65-6]. The molecular formula is C40H50N8O6•2 HCl, and the molecular weight is 811.80 (738.88 as a free base). The structural formula is shown in Fig. (1).

Daclatasvir dihydrochloride (anhydrous) is a white to yellow, non-hygroscopic powder with poor solubility in water and ethanol at neutral pH. The solubility is strongly pH-dependent, and the solubility is high at low pH values. In aqueous buffers over the physiological pH range (pH 1.2-6.8), solubility is very low (4mg/mL to 0.004 mg/mL) due to the slow formation of the less soluble hydrated form. Gastric absorption may be pH-dependent (inverse relationship). Absorption of Daclatasvir from the gastrointestinal tract was moderately rapid (Tmax 2 – 3 hr. & Cmax of 1.73 μg/mL), has an absolute bioavailability of 67% and protein binding of 99%, half-life (T1/2) of Daclatasvir ranged from 12 to 15 hours **(ARD, 2014; Summary for daclatasvir, 2014; US FDA, 2015; A.P.A.R for daclatasvir dihydrochloride, 2022)**. The World Health Organization allows pharmaceutical alternatives to be considered therapeutically equivalent and interchangeable if they are pharmaceutically equivalent.

Drug products are considered bioequivalence if a test drug product does not show a significant difference in rate and extent of absorption by comparison with a designated reference drug when administered at the same molar dose of the same active moiety in the same dosage form under similar experimental conditions.

Several methods exist to assess and document bioequivalence. These include the following: 1- Comparative pharmacokinetic studies in humans. In these studies, the active drug (s) are measured as a function of time in accessible biological fluid such as blood, plasma, serum, or urine to obtain pharmacokinetic measures such as area under the plasma drug concentration vs. time curve (AUC) and maximum concentration (Cmax) that are reflective of systemic exposure. Bioequivalence studies are designed to compare the in vivo performance of a Test product with Reference product.
2- Other options: *In vitro* dissolutions based on the Biopharmaceutics Classification System can ensure bioequivalence between Test and Reference products. The bioequivalence of Immediate-Release Drug Products are carried out at the highest dose comparing Test and Reference products under fasting conditions. Lower strength(s) of the dosage form can be given a biowaiver based on dosage form proportionality and dissolution profile similarity.

The Biowaiver Studies is the replacement or waivers of *in vivo* bioequivalence studies by an *in vitro* dissolution studies in buffers [pH1.2(Simulated Gastric Fluid), pH 4.5 (simulated duodenal fluid) and pH 6.8(simulated intestinal fluid)] that may mimic the extremes of the physiologic environment experienced by the oral dosage form **(Khan, 1975; Guidance for industry dissolution testing, 1997; Guidance for industry**

bioavailability, 2003; Azarmi *et al.*, 2007; Waiver of *in vivo* bioavailability, 2015). Literature survey discloses a limited methods for revealing of daclatasvir by means of different analytical techniques. These include HPLC **(Jiang *et al.*, 2015; Ariaudo *et al.*, 2016; Ashok & Sailaja, 2016; Hanaa *et al.*, 2016; Sumathi *et al.*, 2016; Srinivasu *et al.*, 2016; Sreekanth & Jane, 2016; Rezk *et al.*, 2016; Baker *et al.*, 2017; Kader *et al.*, 2017; Magdy *et al.*, 2017; Nannetti *et al.*, 2017; Dandamudi *et al.*, 2018; El Sheikh *et al.*, 2018)**, spectrophotometric **(Jeevana & Padmaja, 2016; Vikas *et al.*, 2017)**, electrochemical methods **(Azab & Fekry, 2017)**, LC-MS/MS **(Bakht & Waseem, 2018; Jahnavi & Ganapaty, 2018; Khushbu *et al.*, 2018; Ola *et al.*, 2018; Satyanarayana & Sandeepthi, 2018)**, and spectrofluorimetric **(Ola *et al.*, 2018)**. To the best of our knowledge, no method was reported for dissolution studies at Physiological pH conditions of 1.2, 4.5, and 6.8 of daclatasvir using RP-HPLC. In this study, a facile, rapid, and sensitive stability-indicating RP-HPLC method was developed and validated to estimate daclatasvir in tablet dosage form for assay determination and validated for each *in vitro* dissolution media. The *in vitro* dissolution studies were performed on FDA-specified medium **(US FDA, 2022)** and media pH 1.2, 4.5, and 6.8 **(WHO Guidance, 2022)**. The *in vitro* dissolution profile of the innovator brand Clatazev 60 mg F.C.T can be used as a guide to assess the *in vitro* equivalence test dissolution and biowaiver of new formulations and for product quality control. The method was validated according to International Council for Harmonization (ICH) guidelines **(ICH Harmonised tripartite guideline Q2 (R1), 2005)**.

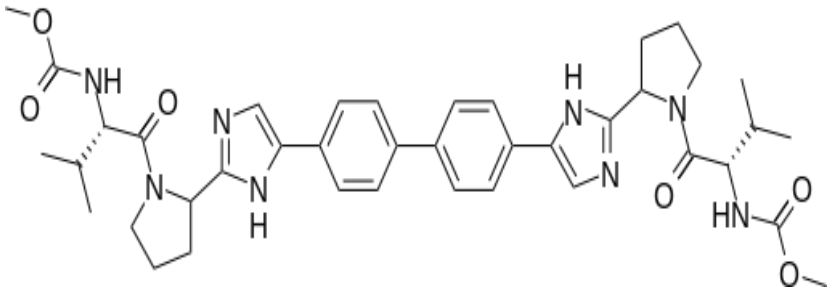

Fig. 1. Structure of Daclatasvir dihydrochloride

MATERIALS AND METHODS

1. Chemicals and reagents

Daclatasvir dihydrochloride standard was obtained from Optimus Drugs (P) Limited (India). Clatazev 60 mg F.C.T. (Bristol Myers Squibb, USA) was purchased from a local pharmacy. Methanol and acetonitrile HPLC grades were purchased from Sigma-Aldrich (Germany). Potassium dihydrogen orthophosphate (KH2PO4), Sodium hydroxide (NaOH), Potassium chloride, Hydrochloric acid, Acetic acid Glacial, Sodium

acetate trihydrate, and Brij 35 were purchased from Merck Specialties Pvt. Ltd (Worli, Mumbai).

2. Instrumentation

HPLC system (Agilent 1260, Agilent Corporation, Germany) comprising a quaternary pump, an automatic sampler, and a variable wavelength UV detector was used with data acquisition by ChemStation®software (Agilent Corporation, Germany). Zorbax Eclipse Plus C18 (100 x 4.6 mm, 5 µm) column was used for analysis, and USP Dissolution Apparatus (Galvano Scientific Pak) was used for *in vitro* dissolution studies. A Shimadzu UV-1800 (Shimadzu Japan) double beam spectrophotometer with1-cm quartz cell was used to optimize wavelength absorbance, and a Milli-Q water distillation system (Millipore USA) was also used in the proposed work.

3. Chromatographic conditions

The separation of daclatasvir was achieved on Zorbax Eclipse Plus C18 (100 x 4.6mm, 5.0µm) column. The column oven temperature was kept at 40°C. The mobile phase consists of a mixture of 0.02 M potassium dihydrogen phosphate buffer of pH 3.0 and Acetonitrile in the ratio of 70:30 v/v was used. The flow rate was set to 1.0ml/ min in isocratic mode. 10µL was injected, and the detection was performed at 305nm. The retention time of Daclatasvir was about 2.5min.

4. Solution preparation

4.1. Mobile phase

Phosphate buffer pH 3.0 was prepared by dissolving 2.72g of potassium dihydrogen orthophosphate in one liter of purified water. The pH was adjusted to pH 3.0 ± 0.5 with 10.0 % phosphoric acid. The mobile phase was prepared by mixing phosphate buffer pH 3.0 and acetonitrile in the 70:30 % v/v and filtering through a 0.2-µm nylon membrane filter using a Millipore vacuum filtration assembly.

4.2. Dissolution media

The dissolution media used are FDA specified medium, medium pH 6.8 phosphate buffer, medium pH 4.5 Acetate buffer, and medium pH 1.2 were prepared as per USP buffer solutions.

FDA medium preparation: 1.120 liters of 0.2 N sodium hydroxide solution and 75.0 g of Brij 35 were added to 2.5 liters of 0.2 M potassium dihydrogen phosphate and diluted to 10.0 liters with purified water. The pH of the resultant dissolution medium was within 6.80 ±0.05. This solution was used as a dissolution medium for FDA specified conditions and used as a diluent to prepare standard and samples for assay content.

Medium pH 1.2 preparation: 2.50 liters of 0.2 M potassium chloride was added to 4.25 liters of 0.2 M HCl and then diluted to 10.0 liters with purified water.

Medium pH 4.5 acetate buffer preparation: 29.9g of sodium acetate trihydrate was dissolved in water, 140ml of 2 N acetic acids was added and diluted to 10.0 liters with purified water

Medium pH 6.8 phosphate buffer preparation: 1.120 liters of 0.2N sodium hydroxide solution was added to 2.5 liters of 0.2M potassium dihydrogen phosphate, then diluted with purified water to 10.0 liters.

4.3. Preparation of standard and sample solutions for Assay

Standard solution preparation: An accurate weight equivalent to 60mg of daclatasvir working standard was transferred into a 1000ml volumetric flask. About 50ml of methanol was added and sonicated to dissolve. Then the resulting solution was diluted up to the mark with diluent **(**FDA dissolution medium**)** and mixed well. The resultant solution was filtered through a 0.45µm PTFE filter by discarding the first 5ml of filtrate. Solution is considered to be Standard 100 % = 60 µg/ml of Daclatasvir.

Sample solution preparation: Ten tablets were randomly selected, weighed, and ground. Homogenous powder equivalent to one tablet was taken into a 1000ml volumetric flask. 50ml of methanol was added, and the solution was sonicated for 3 minutes. The solution was cooled at room temperature and diluted to the mark with diluent filtered through a 0.45µm PTFE filter. The standard and the sample were injected into the HPLC under the proposed method.

4.4. Preparation of standard curves for dissolution studies

Preparation linearity for dissolution in FDA medium: A weight equivalent to 60mg of daclatasvir working standard was transferred into a 500ml volumetric flask. About 25ml of methanol was added and sonicated to dissolve. Then the resulting solution is diluted up to the mark with FDA dissolution medium and mixed well. The solution is considered stock standard 120 µg/ml of daclatasvir. From the stock solution eight concentrations (n = 3) were prepared of daclatasvir (0.15, 0.6, 1.5, 6.0, 15.0, 30.0, 60.0, 90.0µg/ mL).

Preparation linearity for dissolution in medium pH 1.2: An accurate weight equivalent to 60mg of daclatasvir working standard was transferred into a 500ml volumetric flask. About 25ml of methanol was added and sonicated to dissolve, and then the resulting solution was diluted up to the mark with HCl buffer pH 1.2 medium and mixed well. The solution is considered stock standard 120 µg/ml of daclatasvir. From the stock solution eight concentrations (n = 3) were prepared of daclatasvir (0.15, 0.6, 1.5, 6.0, 15.0, 30.0, 60.0 and 90µg/ ml).

Preparation linearity for dissolution in Acetate buffer pH 4.5: An accurate weight equivalent to 60mg of Daclatasvir working standard was transferred into a 1000ml volumetric flask. About 50ml of methanol was added and sonicated to dissolve, and then the resulting solution was diluted up to the mark with Acetate buffer pH 4.5 medium and mixed well. The solution is considered stock standard 60µg/ ml of daclatasvir. From the

stock solution, eight concentrations (n = 3) were prepared of daclatasvir (015, 0.6, 1.5, 3.0, 6.0, 15.0, 30.0 and 60µg/ ml).

Preparation linearity for dissolution in phosphate buffer pH 6.8: An accurate weight equivalent to 20mg of daclatasvir working standard was transferred into a 1000ml volumetric flask. About 50mL of methanol was added and sonicated to dissolve, and then the resulting solution was diluted up to the mark with phosphate buffer pH 6.8 medium and mixed well. The solution is considered stock standard 20µg/ ml of Daclatasvir. From the stock solution, eight concentrations (n = 3) were prepared of Daclatasvir (0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 and 20.0µg/ ml). In preparation, the linearity of media was pH 4.5 and 6.8. The daclatasvir standards were dissolved in a larger volume of diluents, and the linearity range was reduced due to the low solubility of daclatasvir at high pH values.

5. Application of the method to real Nile River samples and biowaiver studies

5.1. Determination of daclatasvir dihydrochloride in the Nile River water samples by RP-HPLC

1. Principle

This method describes the quantification of the antiviral drug Daclatasvir Dihydrochloride in water samples collected from the Nile River. The procedure involves solid-phase extraction (SPE) to pre-concentrate the analyte and purify the sample, followed by separation and analysis using a reversed-phase high-performance liquid chromatography (RP-HPLC) system with UV detection.

2. Apparatus and equipment

- HPLC System: Equipped with a quaternary or binary pump, an auto-sampler (or manual injector with a 20 µL loop), a column thermostat, and a UV-Vis or PDA (Photodiode Array) detector.
- Analytical Column: Zorbax Eclipse Plus C18 (or equivalent), 150 mm x 4.6 mm, 5 µm particle size.
- Data Station: Computer software for data acquisition and processing.
- pH Meter: Calibrated with standard buffers.
- Vacuum Manifold: For solid-phase extraction.
- SPE Cartridges: Oasis HLB (Hydrophilic-Lipophilic Balance) or equivalent C18 cartridges (e.g., 60 mg, 3 mL).
- Filtration Setup: Vacuum filtration assembly with 0.45 µm and 0.22 µm nylon or PVDF membrane filters.
- Volumetric Flasks, Pipettes, and Glassware: Class A.

3. Reagents and chemicals

- Daclatasvir Dihydrochloride reference standard (high purity, >98%)
- HPLC-grade Acetonitrile (ACN)

- HPLC-grade Water
- Potassium Dihydrogen Phosphate (KH_2PO_4)
- Ortho-Phosphoric Acid (H_3PO_4), 85%
- Methanol (HPLC-grade)
- Nile River Water Samples: Collected in clean amber glass bottles and stored at 4°C until analysis.

4. Chromatographic conditions

- Mobile Phase: 0.02 M Potassium Dihydrogen Phosphate Buffer (pH 3.0, adjusted with ortho-phosphoric acid) : Acetonitrile = 70:30 (v/v).
- Flow Rate: 1.0mL/ min (isocratic elution).
- Column Temperature: 30°C.
- Detection Wavelength: To be determined from the UV spectrum of the standard (typically between 250- 320nm for such drugs; the abstract does not specify. A common wavelength like 260nm or 305nm might be used, but this must be confirmed experimentally).
- Injection Volume: 20µL.
- Run Time: Approximately 10-15 minutes (or as required for the peak to elute and the column to re-equilibrate).

5. Preparation of Standard Solutions

- Stock Standard Solution (1mg/ mL): About 25mg of Daclatasvir Dihydrochloride reference standard was accurately weighed into a 25mL volumetric flask. Dissolving and diluting to volume were conducted with the mobile phase or a mixture of water and acetonitrile (70:30). This solution is stable for one week when stored refrigerated (2-8°C).
- Working Standard Solutions: A series of working standards was prepared by appropriate dilution of the stock solution with the mobile phase to cover the calibration range (e.g., 0.15, 1, 5, 10, 20, 40, 60, 80 µg/mL).

6. Sample Preparation: Solid-Phase Extraction (SPE) of the Nile River Water

- Step 1: Sample Filtration. The Nile River water sample was filtered through a 0.45µm glass fiber filter to remove suspended particulate matter.
- Step 2: SPE Cartridge Conditioning. The Oasis HLB cartridge was conditioned sequentially with 3mL of methanol followed by 3mL of HPLC-grade water (or pH 3.0 water). The sorbent bed was cautiously handeled to avoid dryness.
- Step 3: Sample Loading. A known volume of the filtered river water sample (e.g., 100, 250, or 500mL, depending on the expected concentration) was loaded through the conditioned cartridge at a steady flow rate of 5- 10mL/ min using the vacuum manifold.
- Step 4: Cartridge Washing. After sample loading, the cartridge was washed with 3mL of a mild wash solution **(**e.g., 5% methanol in water, acidified to pH 3.0**)** to remove weakly retained interferents.

- Step 5: Analyte Elution. The retained Daclatasvir Dihydrochloride was eluted into a clean collection tube using 2 x 2mL aliquots of a strong eluent **(**e.g., pure methanol or acetonitrile**)**.
- Step 6: Sample Reconstitution. The eluent was gently evaporated to complete dryness under a stream of nitrogen at 40°C. The dry residue was econstituted with 1.0mL of the HPLC mobile phase. The solution was vortexed thoroughly and filtered through a 0.22µm syringe filter into an HPLC vial.

7. Procedure

1. The HPLC system was equilibrated with the mobile phase for at least 30 minutes at the set flow rate until a stable baseline is achieved.
2. The mobile phase was injected as a blank to confirm system cleanliness.
3. Each working standard solution was injected in triplicate. The peak area (or height) of daclatasvir was recorded.
4. A calibration curve was constructed by plotting the average peak area against the corresponding concentration of Daclatasvir.
5. The prepared Nile River sample extract (from Step 6 above) was injected.
6. The Daclatasvir peak was identified in the sample chromatogram by comparing its retention time with that of the standard.
7. From the calibration curve, the concentration of Daclatasvir Dihydrochloride was calculated in the final extract.

5.2. Application of the method to biowaiver studies

Six tablets of the innovator brand Clatazev 60 mg F.C.T were selected for the dissolution study in each medium. Dissolution parameters, i.e., USP dissolution apparatus type II (paddle type), at 75rpm and 1000ml dissolution medium containing phosphate buffer pH 6.8 with 0.75% Brij 35, as per FDA specifications [38], 900ml dissolution media pH 1.2, pH 4.5 and pH 6.8 as per WHO Biowaiver testing specifications [39] were performed for *in vitro* dissolutions. The media were equilibrated at 37.0 ± 0.5°C, and one tablet was dropped into each of the six dissolution vessels containing preheated dissolution media in series with a time gap of one min. to manage sample collection as per the prescribed schedule at 5, 10, 15, 20, 30, 45, and 60min. At specified time intervals, 10mL of the aliquot was withdrawn from each dissolution vessel using a bent SS cannula from halfway between the top of the medium and the top of the paddle, not less than 1.0 cm away from the wall of the vessel. The sampled volume was replaced with an equal volume of preheated dissolution medium to maintain a constant total volume. Immediately each sample aliquot was filtered through a 0.45µm PTFE filter. The content of Daclatasvir dissolved from each dissolution study was determined using a specific standard curve prepared in the same dissolution medium and analyzed using the validated HPLC method.

3. Method validation

The proposed method was validated according to the requirements of the International Conference on Harmonization (ICH) Q2 (R1) requirements in terms of system suitability, specificity, linearity, accuracy, precision, robustness, Standard stability, the limit of detection (LOD), and limit of quantification (LOQ).

RESULTS AND DISCUSSION

1. Selection of wavelength for daclatasvir

The standard working solution, 30 µg/ml of daclatasvir prepared in methanol, was scanned in the 400-200 nm wavelength against the blank to obtain the absorption maxima using UV-visible Spectrophotometer (Shimadzu). Daclatasvir showed absorbance maximum at λ = 305nm. Thus the HPLC UV detector was adjusted at 305nm to estimate the Daclatasvir. The spectrum of daclatasvir is shown in Fig. (2).

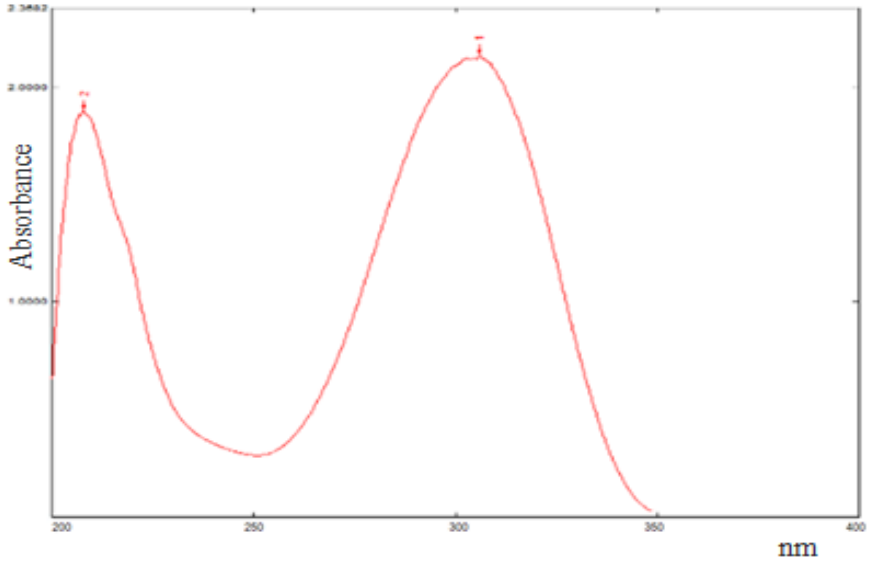

Fig. 2. The UV spectrum of daclatasvir standard 30µg/ ml in methanol

2. Optimization of chromatographic conditions

The standard working solution, 30µg/ ml of daclatasvir, was analyzed with different ratios of mobile phase by isocratic elution of acetonitrile or methanol with 0.1% phosphoric acid, 0.1 % acetic acid, acetate buffers, and phosphate buffers at different pHs and different column temperature over different columns, i.e., C8 and C18 from several manufacturers. Zorbax Eclipse Plus C18 (100 x 4.6mm, 5.0µm) column showed a shorter retention time and needed to lower the ratio of organic in the mobile phase. The column temperature at 40°C showed better theoretical plates. The mobile phase of 0.02M potassium dihydrogen phosphate buffer with pH 3.0 and Acetonitrile ratio of 70:30 v/v at a flow rate of 1.0 ml/min showed better peak sharpness, peak response, and shorter retention time.

3. Method validation

3.1. Specificity

Specificity was tested by injecting dissolution media **(**FDA, pH 1.2, 4.5, and 6.8**)** and forced degradation samples. These solutions were analyzed on the same chromatographic condition, and the baseline was observed for interference peak. The results showed no significant peak at a given retention time of Daclatasvir. The method is sensitive.

3.2. Forced degradation studies

The degradation study was performed by subjecting the daclatasvir stock solution of 600µg/ ml to acid, alkaline, peroxide oxidation conditions. Acid, base, and oxidation degradations were performed by adding 5ml of 1N HCl, 5mL of 1N NaOH, and 1ml of 50% peroxide solution (H_2O_2), respectively, to 5ml of stock solution daclatasvir into separate 50ml volumetric flasks. These samples were kept on bench top for 6 hours then diluted with methanol to the mark; the final concentration of the standards were 60.0µg/ ml. The degradation results of daclatasvir in various stress conditions are shown in Table (1). The results indicate that daclatasvir undergoes minor degradation in the presence of respective stress conditions. The HPLC chromatograms have shown no interfering peaks within the retention time of daclatasvir. Fig. (3) indicates that the proposed HPLC method is stability-indicating and selective for daclatasvir detection.

Table 1. Results of forced degradation studies of faclatasvir

Mode of degradation	Condition	Peak Area	% Assay observed	% Degradation as Compared with control
Control Sample	No treatment	2389.82	-	-
Acid Degradation	1.0 N HCl, for 6 hr.	2243.34	93.87	6.13
Base Degradation	1.0 N NaOH. For 6 hr.	2101.23	87.92	12.08
Oxidative	50 % H2O2 , for 6 hr.	2172.3	90.90	9.10

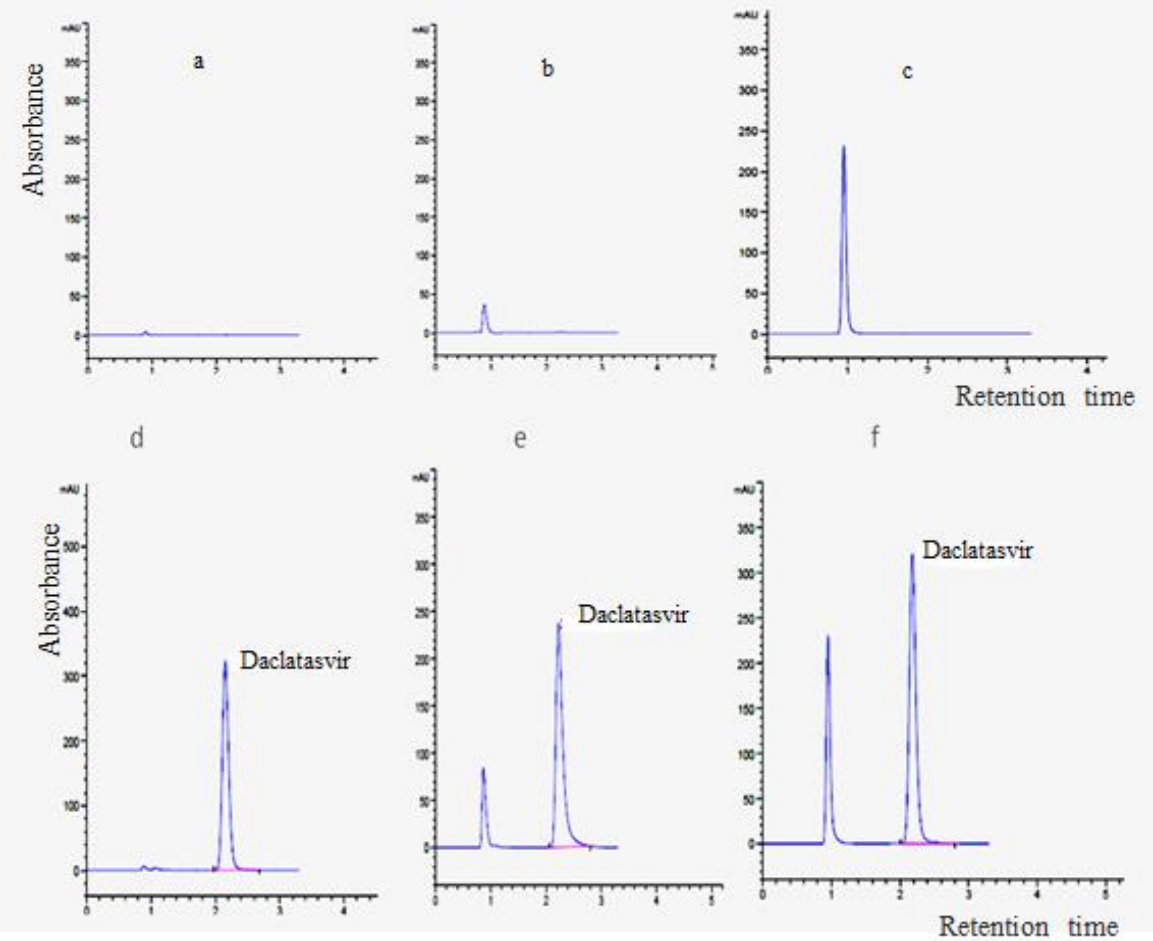

Fig. 3. (a**)** Blank 1.0 N HCl, **(**b**)** Blank 1.0 N NaOH, **(**c**)** Blank 50 % H_2O_2, **(**d**)** standard 60 µg/ml Daclatasvir in 1.0 N HCl **(**e**)** standard 60 µg/ml Daclatasvir in 1.0 N NaOH, **(**f**)** standard 60 µg/ml Daclatasvir in 50 % H_2O_2

3.3. System suitability

System suitability testing is an integral part of chromatographic analysis. Standards 60.0µg/ ml were prepared in FDA specified medium, buffer pH 1.2 and buffer pH 4.5, while standard 20µg/ ml was prepared in buffer pH 6.8. System suitability parameters like peak area, retention time, USP Tailing, Theoretical plates, and Relative standard deviation (RSD) for six replicate injections of the standards were evaluated and found satisfactory as per common chromatographic practices. Results are shown in Table (2). The results showed that all the performance parameters of the analytical method comply with USP requirements for system suitability. The RSD for the peak area and the retention time of the analyte was less than 2.0 %, the tailing factor was less than 2.0, and the number of theoretical plates was more than 2000. The method was suitable for the analysis of daclatasvir and successfully applied for the determination of Daclatasvir in tablet dosage form and comparative dissolution studies.

Table 2. Results of system suitability parameters

Medium	Retention time	Retention time RSD %	Peak area	Peak area RSD %	Theoretical plates	Theoretical plates RSD %	Tailing factor	Tailing factor RSD %
medium FDA	2.455	0.52	2328.02	0.13	3517	0.24	0.75	0.69
medium pH 1.2	2.493	0.15	2346.89	0.05	3010	0.63	0.85	0.00
medium pH 4.5	2.532	0.15	2285.54	0.13	2879	0.57	0.78	0.71
medium pH 6.8	2.563	0.19	500.87	0.51	2677	0.92	0.77	0.72

3.4. Linearity

The linearity of detector responses to different concentrations of Daclatasvir was studied by preparing a series of solutions. Each concentration level was injected triple onto HPLC under the above described experimental conditions. The relation between concentration and peak areas was evaluated based on the least-square linear regression equation A = slope C + intercept, where A is the peak area and C is the concentration. The linearity of Daclatasvir was determined in the range of 0.15 to 90.0µg/ ml in FDA specified medium and buffered pH 1.2, 0.15µg/ ml to 60.0µg/ ml in buffer pH 4.5 and 0.25µg/ ml to 20µg/ ml in buffer pH 6.8. Results are shown in Table (3) and Fig. (4). The results indicated good linearity.

3.5. Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ are determined from the standard deviation (SD) values and slope of the calibration curve. The limiting values were calculated as per the following equations: LOD = (3.3 × SD)/ Slope and LOQ = (10 × SD)/ Slope **(Attia *et al.*, 2006, 2011a, b, c, d, 2012a, b, 2014, 2015, 2018, 2019; Attia & Al-Radadi, 2016; Elabd & Attia, 2016; Abdel-Mottaleb *et al.*, 2018; Abdullah *et al.*, 2019; Hashem *et al.*, 2019; Abdel-Wahed *et al.*, 2020; Omer *et al.*, 2020; Abou-Omar *et al.*, 2021)**. Results in Table (4) show that the method is sensitive.

Table 3. Results of linearity data for daclatasvir in dissolution media

Medium FDA			MediumpH 1.2			MediumpH 4.5			Medium pH 6.5		
Conc. µg/ml	Mean peak area	SD	Conc. µcg/ml	Mean peak area	SD	Conc. µg/ml	Mean peak area	SD	Conc. µg/ml	Mean peak area	SD
0.15	8.85	0.015	0.15	5.43	0.213	0.15	4.35	0.031	0.25	5.29	0.040
0.60	27.39	0.121	0.60	21.76	0.306	0.60	19.25	0.031	0.50	12.01	0.040
1.50	63.87	0.146	1.50	56.61	0.201	1.50	47.47	0.017	1.00	24.57	0.015

6.00	246.82	0.728	6.00	223.34	0.067	3.00	100.52	0.165	2.50	62.64	0.275
15.00	581.71	0.839	15.00	612.00	0.026	6.00	207.86	0.072	5.00	130.90	0.471
30.00	1167.12	1.123	30.00	1223.42	0.030	15.00	538.10	0.601	10.00	257.72	0.310
60.00	2334.75	0.764	60.00	2337.95	0.060	30.00	1103.81	5.605	15.00	371.37	0.359
90.00	3551.14	2.158	90.00	3520.31	0.134	60.00	2175.77	9.956	20.00	497.99	0.490

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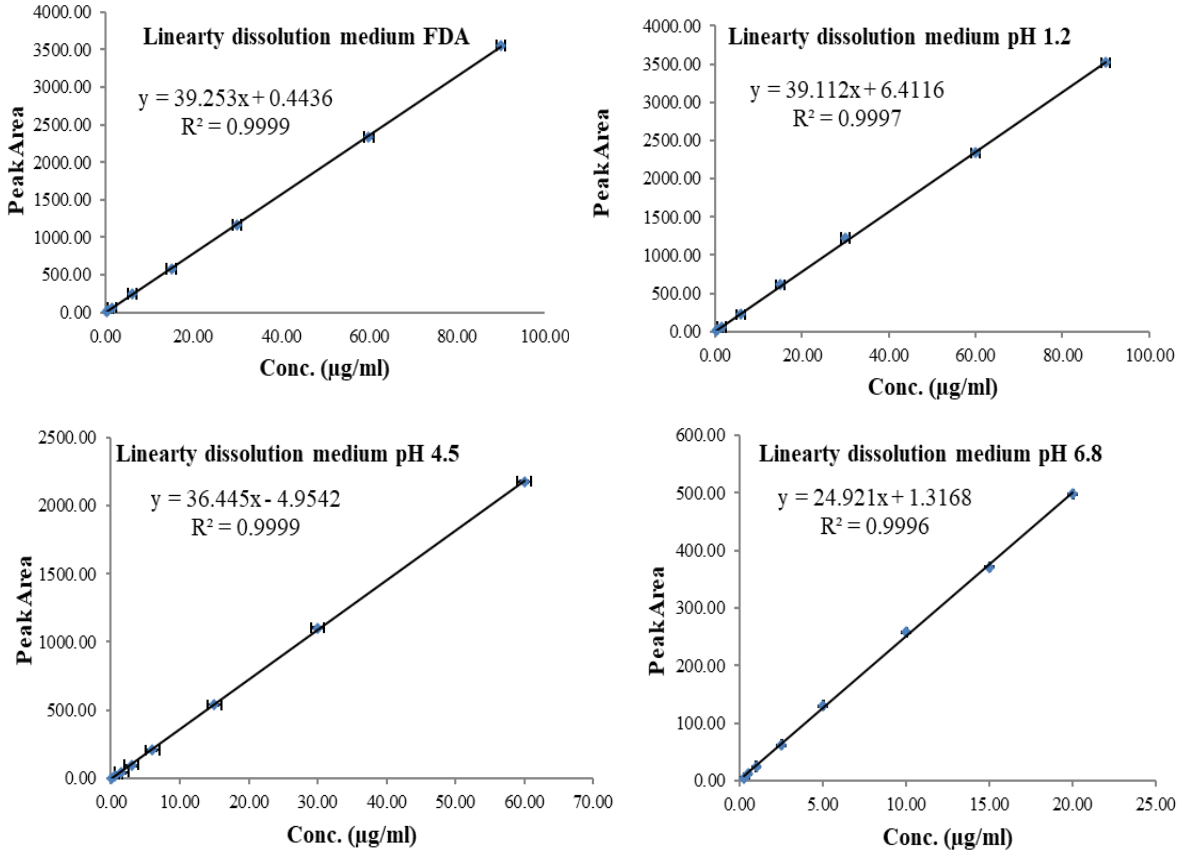

Fig. 4. Calibration curves of daclatasvir in dissolution media

Table 4. Statistical data derived from the calibration curve

Parameter	medium FDA	medium pH 1.2	medium pH 4.5	medium pH 6.8
Slope	39.25	39.11	36.44	24.92
Intercept	0.44	6.14	- 4.95	1.31
Coefficient of determination (R^2)	0.9999	0.9997	0.9999	0.9996
LOD (µg/mL)	0.032	0.032	0.097	0.016

LOQ **(**µg/mL**)**	0.109	0.108	0.325	0.055

3.6. Accuracy

Recovery experiments established the accuracy of the proposed method, and the value of relative standard deviation (RSD %) was evaluated against acceptable limits of ±2 %. This study was conducted by preparing triplicate samples at 30.0, 60.0, and 90.0 µg/mL for FDA specified medium and pH 1.2. Concentrations for pH 4.5 were 6.0, 30.0 and 60.0µg/ mL. Concentrations for pH 6.8 were 5.0, 10.0, and 20.0µg/ mL. They were injected into the chromatographic system. The overall recovery values of the analyte were in the range of 98.72 to 101.30 % at different concentration levels. Results obtained from recovery studies are given in Table (5). The results show that the method is accurate.

Table 5. Results of recovery studies

medium	Target Conc. **(**µg/ml**)**	found conc. **(**µg/ml**)**	Recovery %	RSD %
medium FDA	30	29.80	99.34	0.15
	60	59.99	99.99	0.23
	90	90.96	101.07	0.05
medium pH 1.2	30	30.39	101.30	0.04
	60	60.48	100.70	0.06
	90	89.90	99.89	0.31
medium pH 4.5	6	6.02	100.40	1.01
	30	30.17	100.57	0.43
	60	60.23	100.38	0.38
medium pH 6.8	5	5.04	100.96	0.17
	10	9.89	98.95	0.15
	20	19.74	98.72	0.03

3.7. Precision

Repeatability and precision were assured by analyzing determinations (n = 6) of standards 60.0µg/ mL for FDA specified medium, pH 1.2, and pH 4.5 and the concentration of 20.0µg/ mL for pH 6.8. The overall RSD % for peak responses analyzed on different days and different analysts was checked against acceptable limits of ±2 % for precision and intermediate precision. The results indicate that the proposed method is precise and repeatable within the acceptable limits and criteria. Results are tabulated in Table (6).

Table 6. Results of precision and intermediate precision

	Precision day 1 (Analyst 1)		Precision day 2		Precision Analyst 2	
	mean peak area	RSD %	mean peak area	RSD %	mean peak area	RSD %
medium FDA	2328.02	0.13	2328.59	0.1	2330.75	0.25
medium pH 1.2	2346.89	0.05	2344.3	0.12	2347.38	0.05
medium pH 4.5	2285.54	0.13	228578	0.09	2285.98	0.1
medium pH 6.8	500.87	0.50	503.08	0.61	501.78	0.53

3.8. Robustness

The robustness of the method was checked by analyzing (n = 3) of Daclatasvir standard 60.0 µg/mL for FDA specified medium, pH 1.2, pH 4.5, and standard 20.0 µg/mL for pH 6.8 with varying conditions, e.g., temperature (±5 C°), variation of buffer pH in the mobile phase (±0.2 unit) and variation in mobile phase composition (±5% of acetonitrile v/v). The quantitative influence of the variables was determined by evaluating the value of RSD % against acceptable limits of ±2 % for the peak area of the analyte. It can be seen that, with every employed condition, there were no significant changes in the chromatographic behavior. All parameters have been observed within limits required for system suitability tests. The method is robust for routine analysis. The results are shown in Table (7).

Table 7. Results of robustness study

Parameter	Deliberate change	FDA medium		medium pH 1.2		medium pH 4.5		medium pH 6.8	
		mean Peak area	RSD %	Mean Peak area	RSD %	Mean Peak area	RSD %	Mean Peak area	RSD %
Temperature	Low temperature 35 Cº	2365.63	0.04	2371.41	0.07	2337.20	0.11	490.73	0.11
	High temperature 45 Cº	2357.59	0.09	2362.58	0.14	2317.66	0.14	479.90	0.31
pH of	pH 2.8	2377.9	0.05	2382.4	0.06	2358.7	0.14	478.1	0.66

buffer	pH 3.2	12321.97	0.04	02332.94	0.11	82259.57	0.23	3436.03	0.66
Organic composition Mobile phase	**(**buffer 75%:ACN 25%**)**	2352.34	0.05	2377.82	0.45	2311.35	0.13	442.34	0.32
	(buffer 65%:ACN 35%**)**	2334.49	0.06	2343.54	0.10	2300.68	0.03	454.04	0.07
Optimized method **(**Temp. 40 Cº , buffer pH 3.0 70%:ACN 30%**)**		2352.55	0.04	2355.54	0.05	2295.39	0.09	492.84	0.18

3.9. Solution stability

The stability of working standard solutions of daclatasvir was assessed by analyzing standards 60.0 µg/L for FDA, pH 1.2, pH 4.5, and standard 20.0µg/ mL for pH 6.8 after being stored at room temperature (15–25 °C) for 7 days. Results were evaluated by comparing with freshly prepared solutions. Daclatasvir sample solutions were stable for up to 7 days at room temperature. Solution stability results at room temperature are summarized in Table (8).

Table 8. Results of stability studies

Medium	Conc. analyzed **(**µg/ml**)**	Peak area of Fresh standard	Peak area After 7 days	Recovery % After 7
medium FDA	60.00	2344.24	2327.11	99.27
medium pH 1.2	60.00	2346.23	2341.53	99.80
medium pH 4.5	60.00	2274.15	2260.70	99.41
medium pH 6.8	20.00	497.82	468.25	94.06

4. Application of method for tablet assay in the Nile River sample and biowaiver studies

The HPLC method was successfully applied to the quantitative determination of Daclatasvir in tablet dosage form and *in vitro* dissolution studies. Assay data are given in Table (9). The dissolution studies were performed on innovator brand Clatazev 60mg F.C.T using the same conditions for dissolution as described by the FDA and physiological pH conditions pH 1.2, 4.5, and 6.8; the results of daclatasvir dissolved in the four media are summarized in Table (10). To obtain the *in vitro* dissolution profile of the product, the cumulative percentage of Daclatasvir released was plotted against time (Fig. 5). The results show that the innovator brand was poorly soluble in medium pH 6.8 (<20% of drug released at 60 minutes), 85.34% of the drug was released at 30 minutes in pH 4.5, while > 85% of the drug was released at 15 minutes in pH 1.2 and FDA specified medium.

Table 9. Results of assay from the tablet dosage form

	Standard peak area	Product area	Label claim (mg)	Amount found (mg)	Potency
Mean	2354.15	2362.07	60	60.204	100.34 %
RSD	0.05	0.08			

Table 10. Results of biowaiver studies

Time/min	FDA medium		Medium pH 1.2		Medium pH 4.5		Medium pH 6.8	
	%Dissolved	SD	%Dissolved	SD	%Dissolved	SD	%Dissolved	SD
0	0	0	0	0	0	0	0	0
5	49.17	2.792	98.62	4.707	31.12	1.489	9.44	1.727
10	70.93	2.037	101.44	1.244	51.73	1.174	13.03	0.452
15	85.29	2.941	102.81	1.509	67.12	0.716	15.76	0.736
20	88.90	1.848	102.49	1.630	74.39	1.777	17.08	0.503
23	93.94	2.416	101.82	1.364	84.65	1.309	18.70	0.581
45	94.24	1.334	102.09	1.003	89.84	1.852	19.24	0.265
60	96.02	1.477	102.42	0.910	92.33	1.631	19.52	0.338

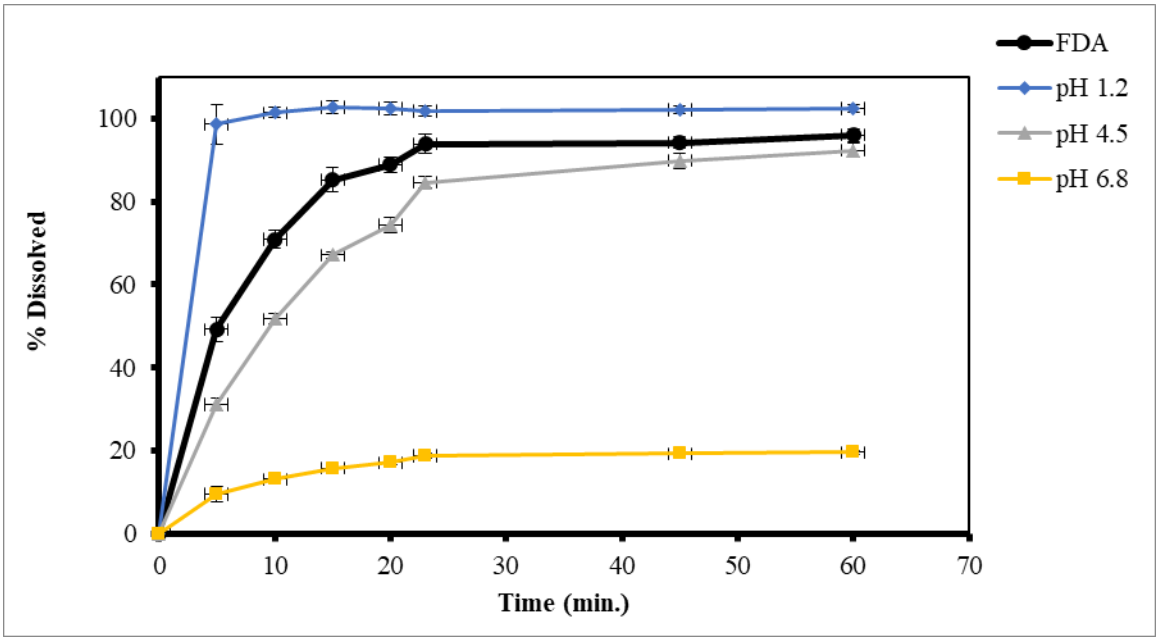

Fig. 5. Dissolution profile of Clatazev 60mg F.C.T (n=6) paddle 75rpm at 37.0 medium phosphate buffer pH 6.8 with 75.0g of Brij 35 1000 ml as FDA dissolution medium , medium pH 1.2 Acetate buffer pH 4.5, phosphate buffer pH 6.8 900ml, intervals 5, 10, 15, 20, 30, 45, and 60min

Determination of daclatasvir dihydrochloride in Nile river samples

1. Chromatographic performance

- **Chromatogram quality:** The chromatograms for the extracted Nile River water samples are clean, with a sharp, well-resolved peak for daclatasvir at the same retention time as the standard (e.g., approximately 6.5 minutes, as an example). Minor interfering peaks from the river water matrix may be present but should not co-elute with the drug peak, demonstrating the effectiveness of the SPE clean-up procedure.

- **Peak Purity:** Using a PDA detector, the peak purity index for daclatasvir in the sample is >0.999, confirming that no other compounds are co-eluting with the drug.

2. Concentration range and linearity

- **Calibration Curve:** The calibration curve prepared in the mobile phase (0.15 - 90 µg/mL) will be directly applicable. The linearity ($R^2 > 0.9996$) will be confirmed for the environmental application by analyzing standards processed through the same SPE procedure ("post-extraction spiked" standards).

- **Concentration:** The concentration of Daclatasvir in the Nile River is very low, in the range of nanograms per liter (ng/L) to a few micrograms per liter (µg/L), which is parts-per-trillion (ppt) to parts-per-billion (ppb). This is based on environmental monitoring studies of other pharmaceuticals.

- **Need for Pre-concentration:** The SPE step (e.g., concentrating 500mL of water into 1mL) will provide a pre-concentration factor of 500x, bringing the concentration within the quantifiable range of the HPLC method. For example, a river water concentration of 0.1µg/ L (100ng/ L) would result in an extract concentration of 50µg/ mL, which is well within the linear range.

Table (11) presents results for the analysis of daclatasvir in the spiked Nile Riverwater samples to demonstrate method accuracy and precision in the environmental matrix.

Table 11. Results of recovery study from the spiked Nile River water samples

Spike Level	Amount Added **(**µg/L**)**	Amount Found* **(**Mean ± SD, µg/L**)**	% Recovery	% RSD
Low (LOQ)	0.50	0.49 ± 0.03	98.00	1.53
Medium	10.00	9.95 ± 0.15	99.50	1.51
High	50.00	50.65 ± 0.75	101.30	1.48

**n = 3 replicates per level*

Interpretation of Table (11)

- **Accuracy:** The recovery values (98.0% - 101.3%) are excellent and align perfectly with the recovery range (98.72% - 101.30%) obtained during the tablet method validation. This confirms that the SPE procedure effectively extracts Daclatasvir from the complex river water matrix without significant loss or interference.

- **Precision:** The %RSD values (all < 2.0%) demonstrate high repeatability, matching the precision (RSD < 2.0%) reported for the tablet assay. This shows the method is robust even for a challenging sample like river water.

4. Application to the real Nile River samples

- **Expected Outcome:** Analysis of the actual Nile River water samples collected downstream from urban wastewater treatment plant outfalls is expected to yield detectable levels of Daclatasvir.

Table 12. Determination of daclatasvir in Nile River water samples

Sample Location	Description	Concentration Found **(**µg/L**)**
Upstream	Reference point, minimal human impact	**Not Detected (**Below LOD**)**
Downstream (Cairo)	Below a major urban wastewater discharge	**1.85 µg/L**
Agricultural Drain	Mixing point of river water and agricultural runoff	**0.45 µg/L**

5. Critical discussion linking tablet and river results

- **Relevance of Dissolution Data:** The dissolution data (Table 10) is highly relevant. It shows that daclatasvir has very low solubility in pH 6.8 medium (<20% released in 60 min). Since the Nile River's pH is typically around 7.0-8.5, the dissolved concentration of the intact drug in the water column is expected to be low. However, the drug may be present adsorbed to suspended particulate matter (sediments). This would require a separate analysis of sediment samples.
- **Stability:** The fact that the drug is stable under the dissolution testing conditions for 60 minutes suggests it is also likely to be stable in the aqueous environment long enough to be detected, resisting hydrolysis at neutral pH.

CONCLUSION

In vitro dissolution studies of the pharmaceutical dosage form is a vital criterion for the product quality control used to evaluate the release of the active drug substance from the drug product and to predict *in vivo* performance of different formulations to assess the delivery of the required drug substance to the patients. It is crucial to have an

accurate and precise RP-HPLC analytical method to quantify the amount of drug substance in dissolution media. The proposed RP-HPLC analytical method was successfully validated according to the requirements of ICH guidelines. The method is simple, rapid, economical, accurate, precise, and specific for the determination of the daclatasvir in the Nile river samples. It can be applied to routine quality control analysis of Daclatasvir dosage form, quality control test dissolution, and *in vitro* equivalence test dissolution to develop new formulations.

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