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METHOD DEVELOPMENT AND VALIDATION OF ASSAY AND DISSOLUTION METHODS FOR THE ESTIMATION OF DACLATASVIR IN TABLET DOSAGE FORMS BY REVERSE PHASE HPLC.

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ABSTRACT

The objective of the present work is to develop a simple, efficient and reproducible stability indicating reverse phase high performance liquid chromatographic (RP-HPLC) method for the estimation of Daclatasvir (DCV) in tablet dosage forms for Assay and Dissolution methods. The developed method for Assay and Dissolution testing of Daclatasvir in tablets was carried out on Zorbax Eclipse Plus C-18 (100 x 4.6 mm, 3.5µm) column using Water and Methanol (20:80) by isocratic run. Flow rate was 1.5 mL min^{-1} with a column temperature of 25°C and detection wavelength was carried out at 315 nm. Total run time for the chromatographic analysis was 2 minutes for both assay and dissolution tests. The forced degradation studies were performed for assay content on Daclatasvir tablets under acidic, basic, oxidation, thermal, humidity and photolytic conditions. No degradation products were observed from the forced degradation studies. The method was validated in terms of specificity, linearity, accuracy, precision and robustness as per ICH guidelines. The method was found to be linear in the range of 10% to 150% of test concentration for assay content and dissolution rate respectively. The percentage recovery values were in the range of 98.6 to 99.8% and 98.0 to 102.6% for assay and dissolution rate at different concentration levels. Relative standard deviations for precision and intermediate precision results were found to be less than 2% and 5% for assay and dissolution rate. The results obtained from the validation experiments prove that the developed method is a stability-indicating method and suitable for routine analysis.

KEYWORDS: Daclatasvir, Assay, Dissolution, Development, Validation, HPLC.

INTRODUCTION

Daclatasvir Dihydrochloride (Daclatasvir) methyl(1S)-1-(2S)-2-(5-(4'- (2-(2S)-1- (2S)-2-(methoxycarbonyl) amino)-3-methylbutanoyl)-2-pyrrolidinyl)-1H-imidazol-5-yl)-4-biphenylyl)-1H-imidazol-2-yl)-1-pyrrolidinyl) carbonyl)-2-methylpropyl)carbamate didihydrochloride is a drug for the treatment of hepatitis C (HCV) virus infection.[1-2] Daclatasvir is a chiral molecule with four stereocenters (1,1', 2, 2;) in the S configuration. Daclatasvir didihydrochloride is synthesised in three main steps using three commercially available well defined starting materials with acceptable specifications. The synthesis involves an alkylation and formation of the imidazole ring, a coupling reaction and the formation of the dihydrochloride salt. Daclatasvir is a white to yellow crystalline non-hygroscopic powder. It is freely soluble in water, dimethyl sulfoxide, methanol; soluble in ethanol (95%); practically insoluble in dichloromethane, tetrahydrofuran, acetonitrile, acetone and ethyl acetate.[2] Daclatasvir structure is shown in Fig. 1.

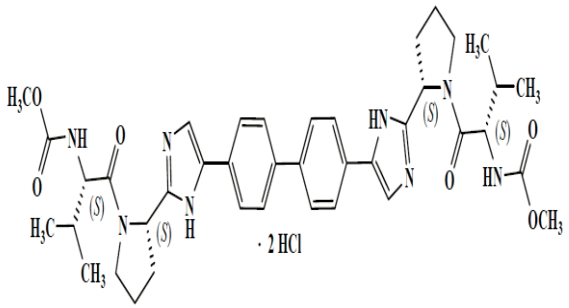

Fig. 1. Structure of Daclatasvir didihydrochloride

Daclatasvir is a first in class direct acting antiviral agent which binds to and inhibits the function of the hepatitis C virus protein NS5A. NS5A is involved in both viral RNA replication and virus particle assembly. A putative inhibitor-binding region spanning amino acids 21 to 30 of NS5A was identified.[2]

The goal of antiviral therapy against HCV is to reach sustained virological response (SVR), which is traditionally defined as the absence of quantifiable virus in plasma at least 24 weeks after the end of therapy.

However, most relapses occur within 4 weeks of treatment discontinuation and a 98-99% concordance has been shown between absence of quantifiable virus 12 weeks after therapy and SVR24. Therefore the absence of measurable virus 12 weeks post end of treatment (SVR12) is presently accepted by European and US regulators as the primary endpoint in clinical trials.[2]

From the literature survey it is evident that very few research articles are available for Daclatasvir. Vinay Sundaram et al. published an article on Dual daclatasvir and sofosbuvir for treatment of genotype 3 chronic hepatitis C virus infection.[3] Paul L. McCormack published a review article on Daclatasvir: A Review of Its Use in Adult Patients with Chronic Hepatitis C Virus Infection.[4] Bunchorntavakul C. et al published a review article on the efficacy and safety of daclatasvir in the treatment of chronic hepatitis C virus infection.[5]

In USP[6] and Ph. Eur.[7], methods are not available for the determination of Daclatasvir in drug substance and drug product. As per the literature review, no method was reported for assay and dissolution study of Daclatasvir by using high performance liquid chromatography (HPLC).

The present research work describes the estimation of assay content and dissolution release of Daclatasvir in tablet dosage forms by using HPLC technique. The work gives a sensitive, specific and stability indicating method for the determination of Daclatasvir in a short run time by HPLC. Methanol is used as solvent for mobile phase preparation as it is less toxic and also available at lesser cost when compared with other solvents like Acetonitrile, Tetrahydrofuran. Developed LC method was validated with respect to specificity, linearity, precision, accuracy and robustness. Forced degradation studies were carried out to verify the stability indicating nature of the LC method.

EXPERIMENTAL

Chemicals and Reagents

Qualified standards (Daclatasvir purity ~99.3%) and tablets are obtained as gift samples from Natco Pharma Limited. HPLC grade methanol (MeOH purity ~99.8%) and water were obtained from Rankem (India). Potassium dihydrogen orthophosphate (KH_2PO_4), Sodium hydroxide (NaOH) and Brij 35 were purchased from Merck specialties Pvt. Ltd (Worli, Mumbai).

Instrumentation

The Waters LC system (Milford, MA, USA) equipped with a diode array detector was used for method development of assay, dissolution rate and for forced degradation studies. The output signal was monitored and processed using Empower software. Waters LC consists of 2695 separation module and 2996 PDA detector used for validation study. Intermediate precision was carried out using waters 2695 separation module with 2996 PDA detector. Photolytic chamber was used for photolytic degradation and thermal degradation samples were kept at 50°C for 3 days in an oven. Dissolution test was carried out using Electrolab dissolution apparatus, system (EDT-08Lx).

Chromatographic conditions

The chromatographic separation was achieved on a Zorbax Eclipse Plus C-18 (100 x 4.6 mm, 3.5µm) column using mobile phase-A composed of Water and mobile phase-B composed of Methanol (20:80) by isocratic program. Flow rate was set to 1.5 mL min^{-1} with a column temperature of 25°C. Detection wavelength was carried out at 315 nm. Injection volume was 10 µL. Water and MeOH in the ratio of 75:25 was used as diluent for the preparation of standards and samples for assay content and dissolution medium was used as diluent for dissolution test.

Preparation of standard and sample solutions for Assay

Preparation of Standard stock solution

Accurately weighed and transferred 33 mg of Daclatasvir working standard into a 100 mL volumetric flask. Added about 70 mL of diluent and sonicated to dissolve with intermittent shaking. The resulting solution is diluted upto the mark with diluent and mixed well.

Preparation of standard solution

Transferred 10 mL of Daclatasvir standard stock solution into a 50 mL volumetric flask and diluted up to the mark with the diluent and mixed well.

Preparation of sample solution

Determined the average weight of 5 tablets and transferred 5 tablets of 60mg equivalent to 300 mg of Daclatasvir directly into a 250 mL volumetric flask. Added about 100 mL of diluent and sonicated for 10 min with intermittent shaking until tablets are completely disintegrated. Added 80 mL of diluent and sonicated further for 45 min with intermittent shaking. Made up the volume to 250 mL volumetric flask with diluent and mixed well. Keep the volumetric flasks on bench top for about 10 minutes to settle down the particulate matter. Transferred the sample solution into centrifuge tubes and centrifuged at 3000 rpm for 15 minutes. Further, transferred 5 mL of above clear supernatant stock solution into a 100 mL volumetric flask and diluted up to the mark with the diluent and mixed well. Transferred the resultant sample solution into HPLC vials for analysis.

Preparation of standard and sample solutions for Dissolution rate

Dissolution parameters

Medium: Phosphate buffer, pH 6.8 with 0.75% Brij 35.
Volume: 1000 ml
Apparatus: USP Type-II (Paddle)
RPM: 75
Temperature: 37°C ± 0.5°C

Time: 10, 15, 20, 30, 45 minutes, Infinity at 200 rpm for 15 minutes.

Preparation of dissolution media
Accurately weighed and transferred 68.0g of KH_2PO_4, 8.9g of NaOH, 75.0g of Brij 35 into 10L of demineralized water. Dissolved the contents and mixed well. The pH of the resultant dissolution medium is within 6.80±0.05.

Preparation of standard stock solution
Accurately weighed and transferred 33 mg of Daclatasvir dihydrochloride working standard into a 100 mL clean dry volumetric flask. To this added 70 mL of dissolution medium, sonicated to dissolve and make up to the final volume with the dissolution medium.

Preparation of standard solution
Transferred 10 mL of the above Daclatasvir standard stock solution into a 50 mL volumetric flask and diluted up to the mark with the dissolution medium and mixed well. Resultant solution is filtered through 0.45µm PVDF filter by discarding the first 7 mL of filtrate and transferred the standard solution into HPLC vials for analysis.

Preparation of sample solution
One tablet was dropped into each of the six dissolution vessels containing preheated dissolution media. 10 mL of the aliquot was withdrawn from each dissolution vessel at the specified time interval (single time point) and replaced with 10mL of preheated dissolution medium (dissolution profile). Sample solution is filtered through 0.45µm PVDF filter by discarding the first 7 mL of filtrate and transferred the solution into HPLC vials for analysis.

Method validation
Specificity/stress studies
Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.[8] The specificity of the developed method was established to prove the absence of interference from placebo peaks (excipients) which is part of required pharmaceutical preparation. Degradation study was performed for assay content analysis by subjecting the tablets to accelerated degradations such as acid, alkaline, oxidation, thermal, humidity and photolytic conditions to evaluate the interference of degradation impurities with main active drug Daclatasvir. Thermal degradation was performed by keeping the tablets in a petri dish and then placed them in an oven at 50°C for 3 days. Humidity degradation was performed by placing the tablets in a petri dish and kept in a humidity chamber at 75% RH, at 25°C for 2 days. Photolytic study was carried out by placing the tablets in a petri dish in a photolytic chamber at 1.2 million lux hour's illumination and 200 watt hours/square meter ultraviolet energy. Acid, base and oxidation degradations were performed by adding 5 mL of 1N HCl, 5 mL of 1N NaOH and 1 mL of 30% peroxide solution (H_2O_2), respectively, to the sample solutions and these samples are kept on bench top for 6 hours. Specificity for dissolution test was performed by injecting the dissolution medium as blank run.

Linearity
Linearity is the ability of the method to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range. The linearity of response for Daclatasvir was determined in the range from 10% to 150% of working concentration for assay and dissolution tests. A graph was plotted between the peak areas versus concentrations to obtain the calibration curve. The seven concentrations of active drug component were subjected to regression analysis by least-squares method to calculate correlation co-efficient and calibration equation. The method of linear regression was used for the data evaluation.

Precision
Precision is a measure of the reproducibility of the whole analytical method under normal operating conditions. The precision was expressed as the relative standard deviation (RSD).
% RSD = (Standard deviation/ average) x 100.

Precision of the developed method was carried out by 6 determinations (preparations) of the test solution by injecting test solution and calculated the % RSD for Assay content and the precision of dissolution rate was determined by preparing the test solution at 100% concentration level for six tablet dosage units and calculated the % RSD for Daclatasvir drug release.

Accuracy
Accuracy or trueness was determined by applying the method to samples in which known amounts of analyte have been added. These should be analyzed against standard and blank solutions to ensure that the sample solution accuracy results are comparable. The accuracy was calculated from the test results as a percentage of the analyte recovered by the assay.

Accuracy of the present method was carried out by injecting the sample solution at three different concentration levels of 50%, 100%, 150% and 10%, 100% and 150% to their test sample concentration, in triplicate determinations for assay and dissolution rate. The %recovery was calculated for the drug added. The mean percentage recovery was calculated.

Robustness
Robustness of the method indicates the reliability of an analysis to assess the system suitability parameters under the influence of small but deliberate variations in method parameters. It was performed by injecting the test solution by changing several parameters including

different batch of the same column, flow rate, column temperature and minor change in organic composition.

Solution stability

Sample solution and the standard solutions containing Daclatasvir were prepared as per the test procedure. All these solutions were divided into two portions. One portion was stored at room temperature and the other portion was stored in the refrigerator at 2-8°C. Freshly prepared solutions and the solutions which were stored at room temperature and refrigerated condition (2-8°C) up to 24 hours were injected at different time intervals. Percent assay and dissolution release obtained at initial was compared with the % assay and dissolution release obtained at different time intervals.

RESULTS AND DISCUSSION

Selection of wavelength for Daclatasvir

Spectra for Daclatasvir was measured from 210 to 400 nm for wavelength maxima. The corresponding spectrum of Daclatasvir is shown in Fig. 2. Based on the spectra maxima, 315 nm was selected for identification and quantification of Daclatasvir in tablets for both assay and dissolution tests.

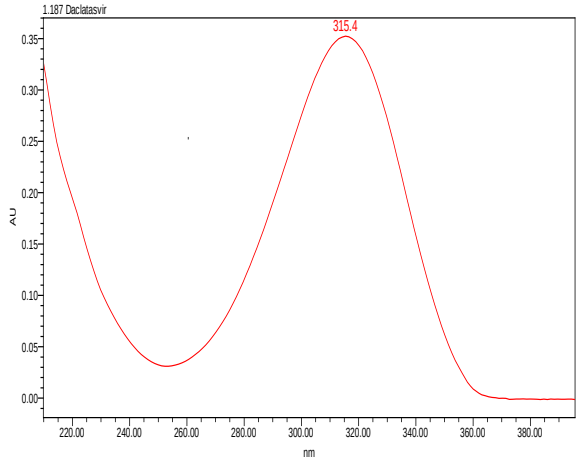

Fig. 2. Spectra of Daclatasvir

Optimization of chromatographic conditions

The main purpose of the current chromatographic method was to develop an LC method[9] for the quantification of Daclatasvir in Daclatasvir tablets for assay content and dissolution rate.

Screening studies were performed on a variety of columns to cover a wide range of stationary phase properties including carbon chain length, carbon loading and surface area. Each of the selected columns was screened with different mobile phase ratios, different column temperatures and the type of organic solvents including MeOH. Trials were performed with mobile phase containing water and methanol (30:70) as solvent with Zorbax Eclipse Plus C-18 (100 x 4.6 mm, 3.5µm) column at a column temperature of 25°C. Flow rate was kept at 1.0mL per minute. Daclatasvir peak eluted at retention time of 3.7 minutes with a tailing factor of 1.0. To reduce the run time, next trial was performed with water and methanol in the ratio of 20:80. Daclatasvir peak is eluted at 1.7 minutes with a tailing factor of 1.1. Final trial was taken with same chromatographic conditions except for change in flow rate from 1.0mL/ min to 1.5mL/ min. Retention time was observed at 1.2 minutes with a tailing factor of 1.1 for Daclatasvir peak at a flow rate of 1.5mL/ min.

Zorbax Eclipse Plus C-18 (100 x 4.6 mm, 3.5µm) column was selected for the final method due to reproducible results and better peak shapes. In most of the trials, Daclatasvir peak shape is symmetrical; however longer retention times were observed. Chromatograms of blank run, standard solution and control sample of Assay content are shown in Figures 3, 4, 5. And also chromatograms of blank run, standard solution and control sample of Dissolution rate are shown in Figures 6, 7, 8.

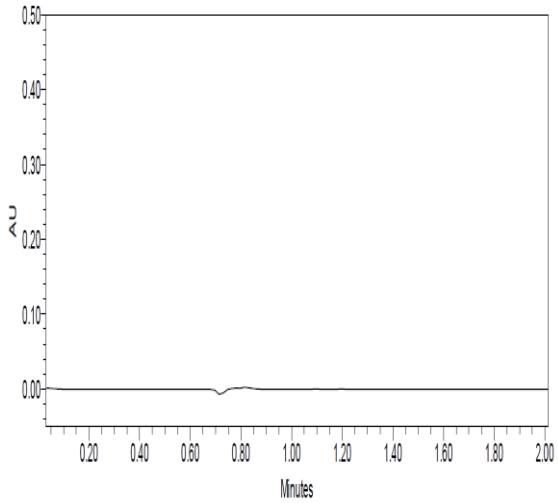

Fig. 3. Blank run of Assay

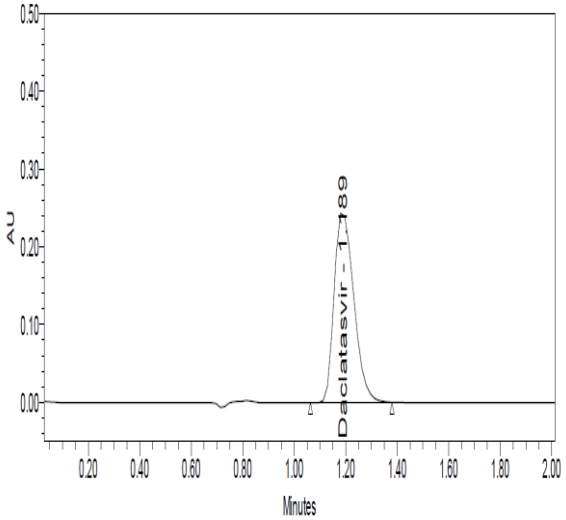

Fig. 4. Standard run of Assay

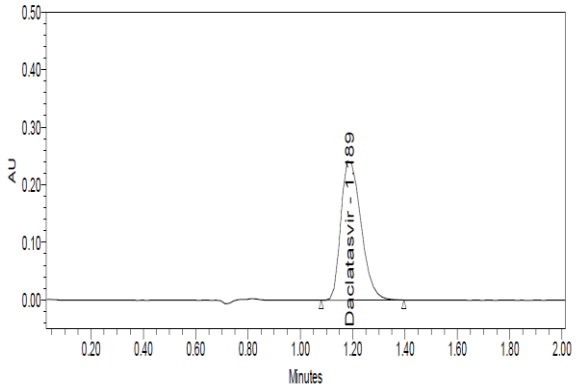

Fig. 5. Sample run of Assay

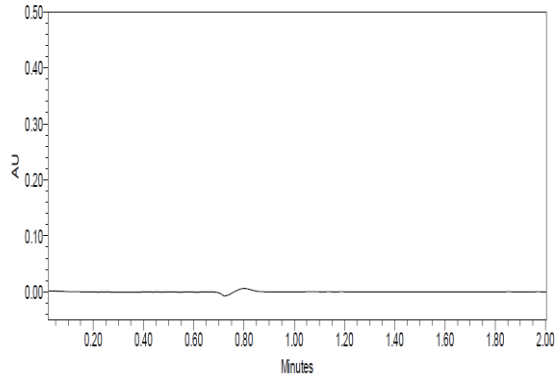

Fig. 6. Blank run of Dissolution test

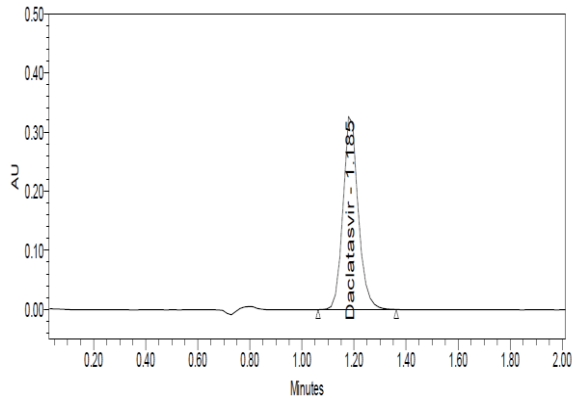

Fig. 7. Standard run of Dissolution test

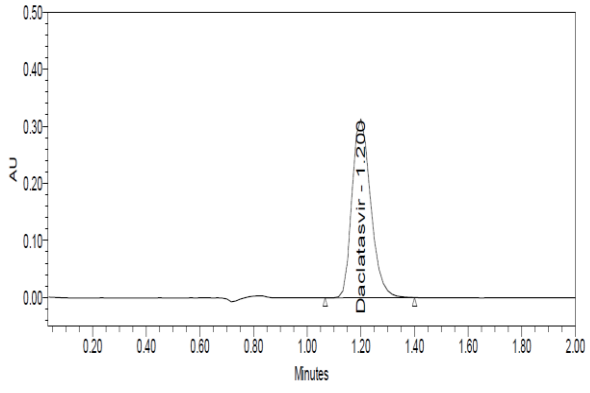

Fig. 8. Sample run of Dissolution test

Method validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended use. The described HPLC method has been extensively validated for both assay and dissolution tests as per ICH guidelines.[8]

After successful completion of method development[9-11], method validation[12–24] was performed to ensure that the developed method was capable of giving reproducible and reliable results when used by different operators employed on the same equipment of the same lab or of different laboratories. Stress testing needs to be performed to elucidate the inherent stability characteristics of the active drug substance and also to prove the stability indicating capability of the method. The developed HPLC method was validated to quantify the Daclatasvir in its tablet dosage form by determining the parameters including specificity, linearity, accuracy, precision and robustness according to the ICH guidelines for assay and dissolution rate.

Specificity

Specificity of the developed method was performed by injecting the Blank solution, Standard solution, stressed degradation samples and the control test solutions for estimation of assay content. The degradation study was carried out using the samples which include tablets containing Daclatasvir and blank solutions. Similarly, specificity for the dissolution rate was carried out by injecting a blank solution and test solutions.

Daclatasvir was found to be stable in all the degradation conditions. Spectral homogeneity of Daclatasvir was checked for purity. Peak purity passed for main active drug Daclatasvir in both standard solution and sample solution. Purity angle value was less than the purity threshold for Daclatasvir peak in standard and sample solutions indicating that Daclatasvir peaks are spectrally homogeneous. Also spectral homogeneity of Daclatasvir peak in degradation samples, found to be similar with those obtained for the standard solution, suggests that no peak was being coeluted at the retention time of Daclatasvir peak. The degradation results of Daclatasvir in various stress conditions were shown in Table 1. The results indicate that Daclatasvir undergoes no degradation in presence of respective stress conditions to form any unknown peaks. No interference was observed from blank solution (dissolution medium) at the retention time of Daclatasvir for the dissolution test.

Table 1. Forced Degradation data of Daclatasvir tablets

Degradation conditions	%Assay observed
Acid treatment (1N HCl, 6 hr)	98.8
Base treatment (1N NaOH, 6 hr)	99.1
Peroxide treatment (30% H_2O_2, 6 hr)	100.7
Thermal-50°C, 3 days	99.2
Humidity-75% RH, 25 °C, 2 days	101.2
Photolytic-1.2 m lux hr, 200 Watt hr/m^2	99.9

Linearity

The linear graphs were plotted between the peak areas versus concentration to obtain the calibration curve. Linearity graphs for Daclatasvir was shown in Fig. 9 and Fig. 10. The response obtained for Daclatasvir was found to be linear from 10% to 150% of standard concentration for both assay and dissolution tests. The correlation co-efficient found for assay and dissolution tests was not less than 0.99 and 0.99. And also statistical values of Daclatasvir were shown in Table 2. The results demonstrate that an excellent correlation between the peak area and concentration of Daclatasvir in tablets for both assay and dissolution tests.

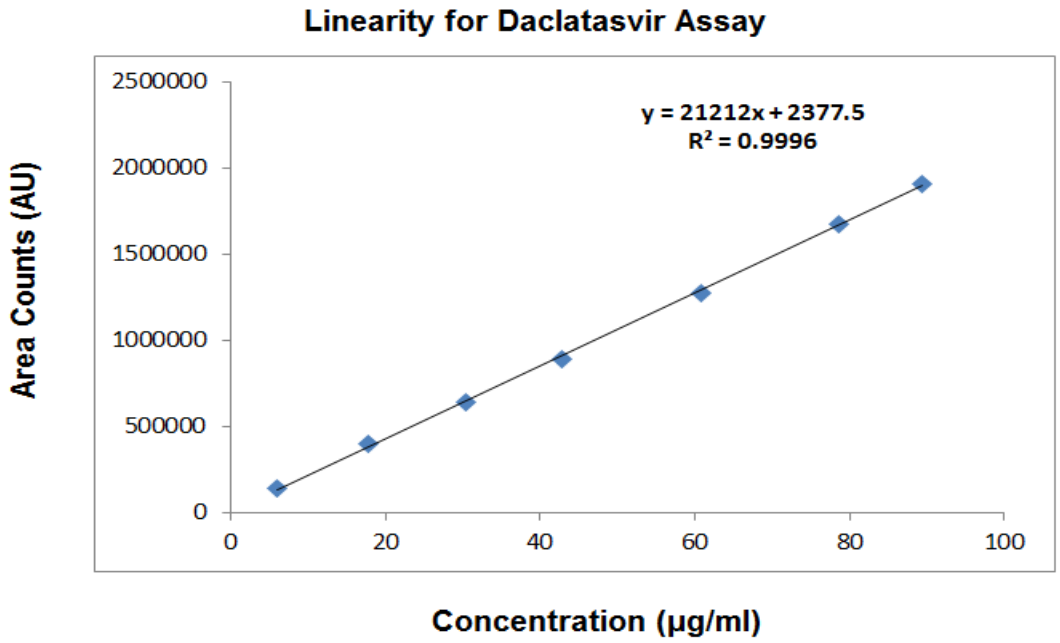

Fig. 9. Linearity graph of Daclatasvir for Assay test.

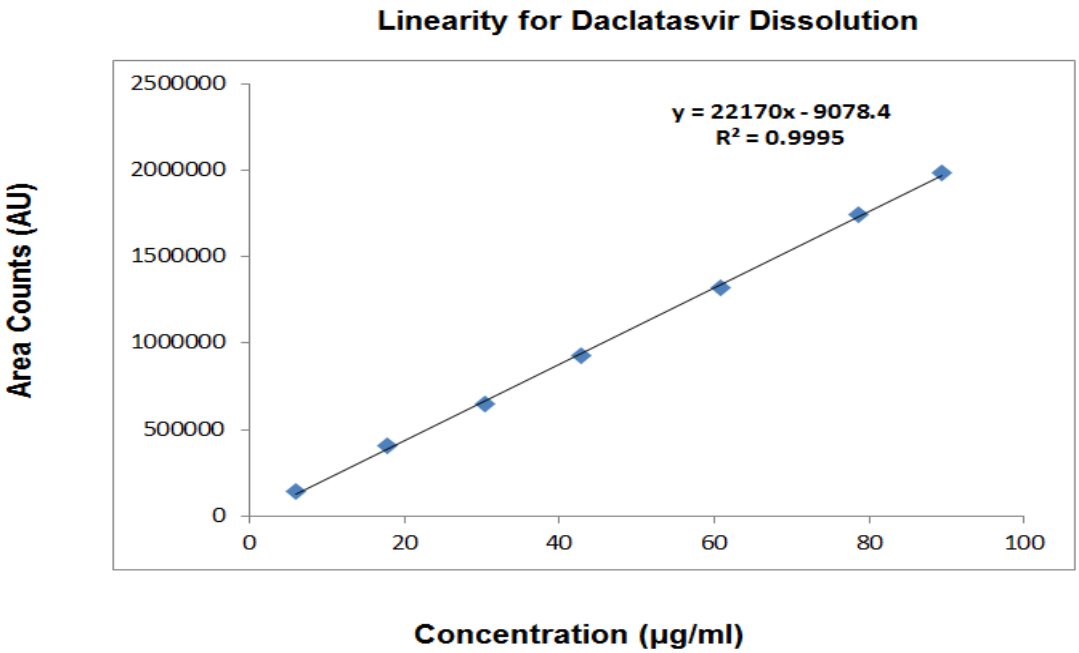

Fig. 10. Linearity graph of Daclatasvir for Dissolution test.

Table 2. Linearity data and regression statistics

Parameter	For Assay	For Dissolution
Slope	21211.79	22170.21
Intercept	2377.45	-9078.42
Coefficient of determination (R^2)	0.9996	0.9995
Intercept at 95% confidence interval (lower value–upper value)	-24525.40– 29280.32	-41571.5– 23414.61
Slope at 95% confidence interval (lower value–upper value)	20720.64– 21702.96	21576.88– 22763.56

Precision

Method precision was determined by injecting 100% test solution of six determinations for assay and dissolution tests and the observed values of % RSD were shown in Table 3. % RSD for Daclatasvir in test solution for six determinations was not more than 2.0% and 5.0% for assay and dissolution tests. Intermediate precision of the method was studied by injecting the test solution of six determinations and the values were shown in Table 3. The %RSD difference between the two analysts is 0.0% for assay and 0.3% for dissolution rate. Less difference between the two analysts shows that the developed method is precise and has good intermediate precision. Dissolution profile results of Daclatasvir at different time intervals of 10, 15, 20, 30, 45 min and infinity time point at 200 rpm for 15 min are shown in Table 4.

Table 3. Precision and Intermediate precision data

Determination	Method Precision (% Assay)	Intermediate Precision (% Assay)	Unit	Method Precision (% Drug Released in 45 min)	Intermediate Precision (% Drug Released in 45 min)
Determination-1	100.46	100.19	Unit-1	104.36	102.69
Determination-2	100.99	100.15	Unit-2	104.59	102.06
Determination-3	100.26	99.86	Unit-3	104.04	102.40
Determination-4	100.34	100.92	Unit-4	103.78	101.23
Determination-5	100.87	100.84	Unit-5	103.54	102.84
Determination-6	101.29	100.36	Unit-6	103.48	103.33
Average	**100.70**	**100.38**	**Average**	**103.96**	**102.42**
SD	0.41	0.41	**SD**	0.44	0.72
%RSD	0.40	0.41	**%RSD**	0.43	0.71

Table 4. Dissolution profile results

	% Drug Released in 10 min	% Drug Released in 15 min	% Drug Released in 20 min	% Drug Released in 30 min	% Drug Released in 45 min	% Drug Released at Infinity (200 rpm for 15 min)
Unit-1	95.40	99.89	101.43	102.47	104.36	105.51
Unit-2	96.29	101.02	102.38	103.56	104.59	105.34
Unit-3	94.96	98.61	100.98	102.61	104.04	105.38
Unit-4	97.17	98.65	100.42	102.15	103.78	105.18
Unit-5	98.52	99.62	101.40	103.00	103.54	104.72
Unit-6	96.33	98.71	100.20	101.90	103.48	104.69
Average	**96.44**	**99.42**	**101.14**	**102.62**	**103.96**	**105.14**
SD	1.27	0.95	0.78	0.59	0.44	0.34
%RSD	**1.33**	**0.96**	**0.78**	**0.58**	**0.43**	**0.33**

Accuracy

The percentage recovery results for Daclatasvir in tablets were varied from 98.6 to 99.8% for assay, 98.0 to 102.6% for dissolution rate at three different concentration levels and the results were shown in Table 5. Based on the % recovery data, it was concluded that the developed method is capable for the estimation of Daclatasvir in tablet dosage form and is adequate for routine analysis.

Table 5. Accuracy results

Accuracy Level for Assay	% Recovery range for triplicate preparations %Assay	Accuracy Level for Dissolution	% Recovery range for triplicate preparations % Dissolution
50%	98.6- 99.5	10%	99.4- 102.6
100%	98.7- 99.8	100%	98.0- 99.0
150%	99.0- 99.1	150%	99.3- 99.7

Robustness

In all the robust conditions (flow rate, column temperature, organic composition change in mobile phase and change of other column), the tailing factor for Daclatasvir was less than 2.0. Retention time (RT) and tailing factor values for different robustness parameters were shown in Table 6 for assay and dissolution tests. The peak shapes for Daclatasvir in different robustness conditions were found to be symmetrical. Peak purity for Daclatasvir peaks were also tested to observe no interference observed in all the robustness conditions for assay content.

Table 6. Robustness results

Robustness Parameters	For Assay		For Dissolution	
	Retention time in minutes	Tailing factor	Retention time in minutes	Tailing factor
Control Sample	1.19	1.28	1.20	1.23
Using other batch column	1.19	1.27	1.20	1.21
Flow rate (1.6 mL/min)	1.12	1.39	1.12	1.34
Flow rate (1.4 mL/min)	1.27	1.38	1.27	1.37
Low column temperature 20°C	1.19	1.31	1.20	1.36
High column temperature 30°C	1.19	1.31	1.20	1.32
Minor component (Water: Methanol: :25:75)	1.58	1.37	1.60	1.36
Major component (Water: Methanol: :15:85)	0.99	1.35	0.99	1.24

Solution stability

The percent difference of Daclatasvir was determined for solutions stored at room temperature and at refrigerated condition in different time intervals up to 24 hours for assay and dissolution tests. Daclatasvir sample solutions were found to be stable up to 24 hours at room temperature and also at refrigerator condition. Solution stability results at room temperature and refrigerator conditions were shown in Table 7.

Table 7. Solution stability results of standard and control sample at 2-8°C and room temperature

For Assay	Time interval		
	Initial	After 24 Hours	% Difference
% Assay of standard solution at RT	100.3	99.5	0.8
% Assay of sample solution at RT	100.5	100.1	0.4
% Assay of standard solution at 2-8°C	100.3	100.1	0.2
% Assay of sample solution at 2-8°C	100.5	100.2	0.3
For Dissolution			
% Assay of standard solution at RT	100.6	100.2	0.4
% Assay of sample solution at RT	104.4	103.5	0.9
% Assay of standard solution at 2-8°C	100.6	100.1	0.5
% Assay of sample solution at 2-8°C	104.4	103.8	0.6

CONCLUSIONS

A novel RP-HPLC method was developed for the estimation of assay content and dissolution rate of Daclatasvir in its pharmaceutical dosage forms. Degradation behavior of Daclatasvir was studied under various degradation conditions. No degradation peaks were observed in the respective stress conditions. Daclatasvir peak shape is symmetrical and no interference from unknown degradation impurities was observed which shows the stability-indicating capability of the method. The developed method can be used for the quantification and dissolution rate of Daclatasvir in tablet dosage forms in routine analysis.

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