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STABILITY INDICATING HPLC METHOD DEVELOPMENT AND VALIDATION FOR DETERMINATION OF DACLATASVIR IN PURE AND TABLETS DOSAGE FORMS.

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Abstract:
A sensitive, simple, selective and accurate HPLC method was developed and validated for analysis of antiviral drug Daclatasvir (BMS-790052, DCV) in pure form and in tablet dosage form in the presence of its degradation products. The chromatographic separation achieved by isocratic elution on Hypersil BDS C18, 4.6×150 mm, 5µm column at 25˚c. The mobile phase was a mixture of 0.05M Potassium dihydrogen phosphate (pH-4.5) and acetonitrile in ratio of 50:50 (v/v). The injection volume was 10µl. The flow rate was 1ml/ minute. The detection wavelength was 320 nm. The developed method was validated as per ICH guidelines; it was precise, accurate and robust. The calibration curve of Daclatasvir was linear in range 0.5- 100µg/ml with a correlation coefficient ≥ 0.999. Also the validated method was helpful for rapid routine analysis as the run time was less than 3 minute; the retention time for Daclatasvir was about 2.33 minute. The method was successfully applied to analysis of Daclatasvir in tablet form and the recovery was from 99.71% to 100.86%.
Keywords: *HPLC, stability indicating, Daclatasvir, tablets.*

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INTRODUCTION:

The hepatitis C virus (HCV) is chronically infect about 150-200 million people worldwide and up to 350000 people die every year from hepatitis related diseases. HCV prevalence varies greatly, but the highest prevalence (15–20%) has been reported from Egypt. The treatments in past was consisted of a combination of pegylated interferon alpha and antiviral drug ribavirin but this medication effect was very low particularly for genotype 1 HCV, the cure rate was about 45%[1, 2].

The discovery of the new RNA polymerase inhibitor boceprevir, telaprevir, sofosbuvir and daclatasvir provide a high cure rate giving an option for patient who has previously failing therapy. Two first generation direct-acting antiviral (DAA), telaprevir and boceprevir, were approved in the US and EU in 2011 for the treatment of GT-1 chronic hepatitis C (CHC). However, both of these agents must be co administered with interferon (IFN) and ribavirin (RBV), and are therefore associated with the known adverse effects of the IFN/RBV backbone, potentially limiting their overall effectiveness. Developing of a new generation IFN/RBV-free DAAs that can improve efficacy and safety in a broader GT population of CHC-infected subjects remains a high priority [3, 4] .

Daclatasvir is a novel nonstructural protein 5A (NS5A) inhibitor recently approved in the USA for treatment of genotype 3 HCV infection (GT3). GT3 HCV has been difficult to treat with direct-acting antiviral. Daclatasvir has an increasing database of evidence to show its efficacy against this genotype [5].

Daclatasvir(Fig.1)Methyl[(2S)-1-{(2S)-2-[4-(4'-{2-[(2S)-1-{(2S)-2-[(methoxycarbonyl)amino]-3-methylbutanoyl}-2-pyrrolidinyl]-1H-imidazol-4-yl}-4-biphenylyl)-1H-imidazol-2-yl]-1-pyrrolidinyl}-3-methyl-1-oxo-2-butanyl]carbamate is one of the highly potent and selective DAAs of HCV non-structural (NS) proteins[6]. Few methods were reported for Daclatasvir determination such as LC-MS/MS[7-9] .No reported method was found based on HPLC-UV for Daclatasvir .The proposed method was helpful in routine work and helpful in quick analysis of a large number of samples in short time. Thus in this research a simple, sensitive and direct analysis without complicated sample preparation HPLC-UV method was optimized and validated for Daclatasvir determination in pharmaceutical dosage form.

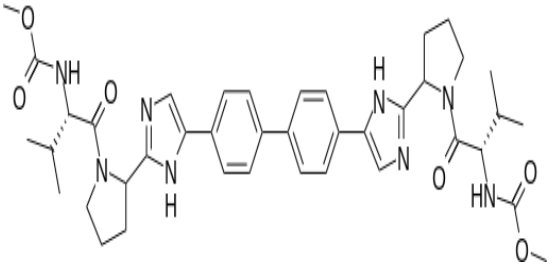


Fig.1: Daclatasvir structure

EXPERIMENTAL:

Instrumentation: Agilent HPLC1260 Infinity Quaternary LC System, auto- sampler, DAD UV-VIS detector, using Chemstation software was used for the analysis .The reversed stationary phase was Hypersil BDS, C18, 4.6×150mm, 5µm column at 25˚c..All pH measurements were carried out on Jenway 3510 pH meter. Shimadzu ATY 224 analytical balance was used for all weighing process.

Material and chemical reagents: HPLC grade acetonitrile obtained from J.T. Baker, United States. Sodium hydroxide pellets and hydrochloric acid 36.5% from Scharlau, Spain. Hydrogen peroxide 30% from Merck KGaA, Germany. Extra pure potassium dihydrogen orthophosphate obtained from Lobachemie, India. Daclatasvir dihydrochloride working standard obtained from Topharman Shanghai Co., ltd, china. Andodaclta 60 mg film coated tablets, Al-andalous for pharmaceutical industries, Egypt. Bi-distilled water was used throughout all work.

Preparation of solutions:

***Preparation of 0.05 M phosphate buffer*:** 6.8 g Potassium dihydrogen orthophosphate were weighed and transferred to 1000 ml beaker, dissolved and diluted to 1000 ml with water .The pH finally adjusted to 4.5 using Ortho-phosphoric acid.

Preparation of mobile phase: A mixture of 0.05M Potassium dihydrogen orthophosphate (pH-4.5) and acetonitrile in a ratio 50: 50 (v/v) was prepared, degassed and filtered under vacuum through 0.45 µ nylon membrane filters**.**

Preparation of stock and working standards: A stock solution of Daclatasvir (60µg/ml) was prepared by dissolving 65.92 mg of Daclatasvir dihydrochloride (equivalent to 60 mg daclatasvir) working standard in 50% methanol into 1000 ml volumetric flask. The volume was completed to the mark with the same solvent. Working solutions were prepared by diluting the stock solution with 50% methanol using suitable volumetric pipettes to prepare a solution in concentration ranging from 0.5 to 100 µg/ml to study the linearity and range of the method. Solutions in concentration ranging from 0.6 to 6 µg/ml were used to study the validation parameters.

Preparation of sample solution: Ten tablets (Andodaclta 60 mg film coated tablets) were weighed and the average weight was calculated then ground to a fine powder. A stock solution (60µg/ml) was prepared by weighing 380 mg from the ground powder (equivalent to 60 mg of Daclatasvir) .The weighed sample was then transferred into 1000 ml volumetric flask, dissolved with 50% methanol then the content of the flask was sonicated for 10 minutes to achieve complete dissolution then cooled to room temperature. The volume was then completed to the mark with 50% methanol. The solution was then filtered through

0.45µm filter, the first portion of filtrate was rejected, then 10 ml from the filtrate was accurately transferred to 100 ml volumetric flask and the volume was then completed to the mark with 50% methanol**.**

Preparation of a placebo solution: A stock solution was prepared by weighing 380 mg from synthetic mixture of the drug product components (placebo).The weighed sample was then transferred into 1000 ml volumetric flask, dissolved with 50% methanol then the content of the flask was sonicated for 15 minutes to achieve complete dissolution then cooled to room temperature. The volume was then completed to mark with 50% methanol. The solution was then filtered through 0.45µm filter, the first portion of filtrate was rejected, then 10 ml from the filtrate was accurately transferred to 100 ml volumetric flask and the volume was then completed to mark with 50% methanol**.**

Preparation of forced-degradation solution: A stock solution of Daclatasvir (100µg/ml) was prepared by dissolving 109.87 mg of Daclatasvir dihydrochloride working standard (equivalent to 100 mg daclatasvir) in 50 ml methanol into 1000 ml volumetric flask. The volume was completed to the mark with purified water .Volumes of 10 ml of Daclatasvir stock solution were transferred into 100 ml volumetric flasks. Volumes of 5 ml of 1N HCl or 10 ml 1 N NaOH were added and the mixtures were kept at room temperature for 8 hr. Similar reaction mixtures were prepared in test tubes and were placed in a water bath at 70˚C for 1 h. During heating, volume loss was compensated with purified water. After the specified time intervals, the mixtures in the test tubes were quantitatively transferred into 100 ml volumetric flasks. All solutions were neutralized with appropriate volumes of NaOH or HCl and diluted to volume with 50% methanol to reach final concentrations of 10 µg/ml Daclatasvir.

For the oxidative degradation solution, a volume of 10 ml of Daclatasvir stock solution was transferred into a 100 ml volumetric flask. A volume of 10 ml of H_2O_2 30% was added and the mixture was kept at room temperature for 24 h. Another similar reaction mixture was prepared in a test tube and was placed in a water bath at 70 ˚C for 3 hr. After the specified time interval, the mixture in the test tube was quantitatively transferred into a 100 ml volumetric flask, and then both solutions were diluted to volume with 50% methanol to reach final concentrations of 10 µg/ml Daclatasvir.

Chromatographic conditions: The analysis was achieved on a reversed stationary phase Hypersil BDS, C18, 4.6×150 mm, 5µm column at 25˚c. The mobile phase was a mixture of 0.05M Potassium dihydrogen orthophosphate (pH-4.5) and acetonitrile in a ratio 50: 50 (v/v). The flow rate was 1 ml/ min .The injection volume was 10µl .the UV detection wavelength was 320 nm. A freshly prepared mobile phase was passed on the column for 15 min. before injection.

Method validation: Validation of the method was carried out according to ICH guidelines[10] .

Specificity: Specificity was assessed by injection of a placebo solution and forced degraded solutions by acid (1N HCl), base (1N NaOH) and oxidizing agent (30% H_2O_2) and observes that no interference between Daclatasvir peak and other peaks present in the chromatogram.

Precision: precision was done for three level of concentration, each concentration repeated three times in the same day for intraday precision and then the procedure repeated in another day for inter day precision.

Linearity: linearity of the method was tested for ten concentrations in a range from 1 to 100 µg/ml and the area under the peaks were plotted against the concentrations; a good linear curve was obtained with a regression coefficient close to 1.

Accuracy: accuracy of the method was tested by % recovery of Daclatasvir on three concentrations; each concentration was repeated three times. Known amounts of Daclatasvir were added to a synthetic mixture of the drug product components (placebo) and subjected to analysis procedure. The % recovery was then calculated

Ruggedness: ruggedness was tested for different analytical conditions like different day and analyst to measure the capability of the method to remain unaffected by these variations that expected during method usage.

Robustness: The method was evaluated within small variation in its parameter and was found to be robust. Robustness was examined by small change in the flow rate (±0.1ml/min), pH of phosphate buffer (±0.05) and percentage of organic solvent (±1%).

Stability: stability of prepared samples was tested for 7 days when stored away of light at 5 & 25 ˚c and found to be stable for this period.

LOD& LOQ: The limit of detection (LOD) and the limit of quantification (LOQ) were determined by injecting a series of samples of low concentration and from the calibration curve the LOD and LOQ were estimated as per ICH guidelines.

RSULTS AND DISCUSSION:

Method development &optimization: The goal of our research is development of a simple, accurate, rapid and reliable for quick analysis method. One of the most important aspects to achieve this goal is to get acceptable resolution with best peak symmetry and reasonable analysis time.[11,12]

Before development of HPLC method for daclatasvir, important information about its properties was collected. The solubility was found to be highly in 50% methanol so 50% methanol was selected as solvent for preparation of all solutions.

From the spectrum of Daclatasvir, Daclatasvir was showing ƛ max at 320 nm, so the wavelength 320 nm was selected for all measurements.

From the study of the structure of daclatasvir, it was clear that it has good retention in the reversed phase column so among reversed phase c18 and c8 with different lengths, the c18 was selected as it give the best retention & symmetric and sharp peak with less tailing factor.

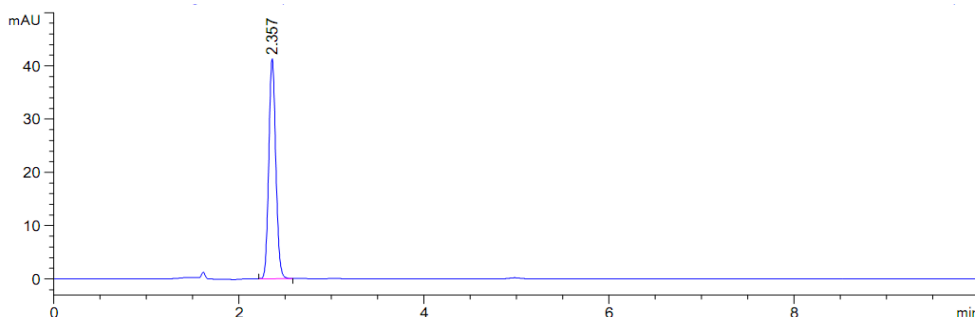
Several mobile phases were tried through change of mobile phase composition, buffer type, buffer pH and organic modifier.

In initial trials water ,acetate buffer and phosphate buffer were tried with different concentration of organic modifier and it was observed that water and acetate buffer decrease peak sharpness and theoretical plates so phosphate buffer was selected for the best peak sharpness and plates .

mixtures of phosphate buffer with different concentration of organic solvent (acetonitrile and methanol) were tested and it was observed that methanol was not preferred as it increase analysis time and give less sharp peaks as acetonitrile so a mixture of phosphate buffer and acetonitrile in ratio of 50: 50 (v/v) was selected as mobile phase which gave us the best beak with a reasonable retention time.

Phosphate buffers with different pHs from 2.5 to 6.5 were tested. Daclatasvir peaks in case buffer with pH value higher than 4.5 were wider, less sharp and the theoretical plates were fewer. When pH was decreased below 4.5 the peak splited and distorted.

Finally, among these mobile phases a mixture of 0.05M Potassium dihydrogen phosphate (pH-4.5) and acetonitrile in ratio of 50: 50 (v/v), injection volume 10µl, flow rate 1ml/ min on Thermo hypersil BDS C18, 4.6×150mm, 5µ at 25˚c and UV detection wavelength 320 nm was selected as optimum for the best peak symmetry, theoretical plates and retention time fig.2 .



RetTime [min]	k'	Area [mAU*s]	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
2.330	-	201.81624	39.97259	0.88	0.0780	4943	-	-

Fig. 2: Daclatasvir HPLC chromatogram

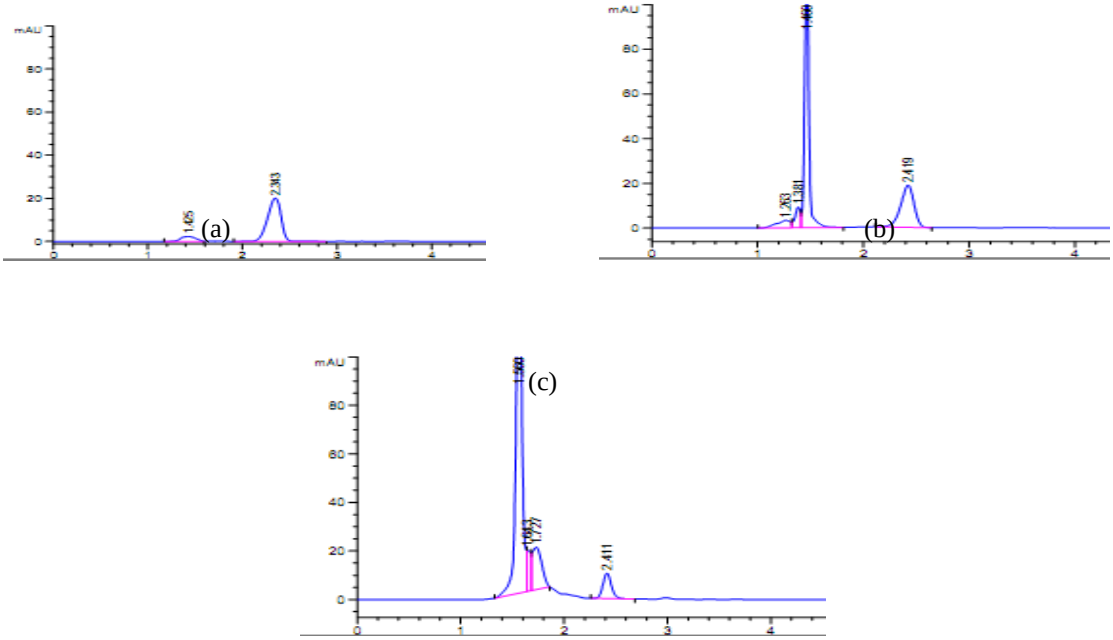


Fig. 3: degradation of Daclatasvir by (a) acid (1N HCl) ,(b) base (1N NaOH) , (c) oxidizing agent (30% H_2O_2)

Method validation

Validation of the method was carried out according to ICH guidelines [10] to ensure that the method is suitable for its intended use. Specificity, accuracy, precision, linearity, ruggedness and robustness all these parameters were tested and were found in acceptable limits.

Specificity: Specificity was assessed by injection of a placebo solution and forced degraded solutions and found there no interference between Daclatasvir peak and other peaks present in the chromatogram. So the method is selective for Daclatasvir and can differentiate it from other peak may be present in the chromatogram Fig. 3.

Linearity: Linearity of the method was tested for ten concentrations in a range from 1 to 100 µg/ml Table1 .The areas under the peaks were plotted against the concentrations. Linear relationship was observed Fig 4. The regression parameters have been shown in Table 2.

Table 1- Linearity of Daclatasvir

Concentration(µg/ml)	**Peak area**
1	34.71
5	173.58
8	271.00
10	339.19
20	671.02
50	1672.55
60	2003.46
70	2347.94
80	2691.21
100	3339.09
*each result is the average of three determinations	

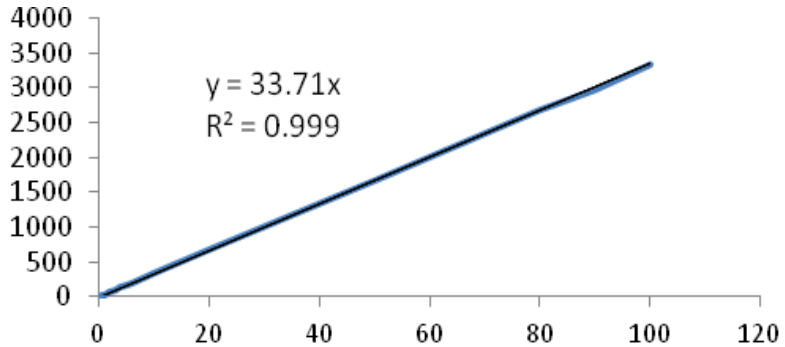


Fig.4 daclatasvir range from 1 to 100 µg/ml

Table 2- regression parameter obtained from Daclatasvir linearity curve	
parameter	
Range(µg/ml)	1-100
Slope	33.433
Regression coefficient(r)	0.9999
Square Regression coefficient(r2)	0.9999
Intercept	3.67

Precision (repeatability):
Precision of the method was tested for three concentrations (1.5, 3 & 6 µg/ml)/ three replicate each. Percent of the relative standard deviation for each concentration was shown in table 3.

Accuracy:
The accuracy of the method was determined by the % recovery over three level of concentration (1.5, 3 & 6 µg/ml).Known amounts of Daclatasvir were added to a synthetic mixture of the drug product components (placebo) and subjected to analysis procedure. The method accuracy range was from 99.71% to 100.86%. The percent of recovery and % RSD was shown in table 4.

Ruggedness:
The reproducibility of the method was evaluated under different conditions (different analysts and days). The analysis of the same samples was repeated by different analyst and in different day .percent of recovery and the RSD were calculated and shown in table 5 and table 6.

Table 4- Recovery study for Daclatasvir Tablets showing accuracy and precision of the analytical method for assay

	%Recovery of Daclatasvir		
Sample No.	**1.5 µg/ml**	**3 µg/ml**	**6 µg/ml**
1	100.26	100.67	99.81
2	100.49	100.09	99.71
3	100.76	99.99	99.90
Mean	100.50	100.25	99.81
%RSD	0.25	0.36	0.10

Table 5- Ruggedness (different analysts)

	%Recovery of Daclatasvir	
Sample No.	**first analyst**	**second analyst**
1	100.00	100.02
2	100.07	100.04
3	100.12	100.05
Mean	100.06	100.04
%RSD	0.06	0.02

Table 6- Ruggedness (different days)

	%Recovery of Daclatasvir	
Sample No.	**first day**	**second day**
1	99.86	99.95
2	99.93	99.64
3	99.93	99.88
Mean	99.91	99.82
%RSD	0.04	0.16

Table 7- Robustness

	Peak area (response)		
Sample No.	**Flow rate 0.99**	**Flow rate 1.0**	**Flow rate 1.01**
1	208.90	205.58	204.79
2	208.89	205.72	204.93
3	208.89	205.82	204.73
Mean	208.89	205.71	204.82
Pooled mean	206.4722		
SD	1.857968		
%RSD	0.90		

Table 8- Robustness

	Peak area (response)		
Sample No.	**Acetonitrile 49%**	**Acetonitrile 50%**	**Acetonitrile 51%**
1	206.89	205.58	207.16
2	207.14	205.72	207.04
3	206.32	205.82	207.15
Mean	206.78	205.71	207.12
Pooled mean	206.54		
SD	0.675642		
%RSD	0.33		

Table 9- Robustness

	Peak area (response)		
Sample No.	**pH4.45**	**pH4.5**	**pH4.55**
1	207.31	205.58	207.24
2	207.13	205.72	207.29
3	207.56	205.82	207.54
Mean	207.33	205.71	207.36
Pooled mean	206.80		
SD	0.831685		
%RSD	0.40		

Robustness: The method was evaluated within small variation in its parameter and was found to be robust. Robustness was examined by small change in the flow rate (±0.1ml/min), pH of phosphate buffer (±0.05) and percentage of organic solvent (±1%). These changes didn't have significant effect on peak area or retention time. The result was shown in table 7,8 & 9.

Stability: Stability of the prepared samples was tested for 7 days when stored in a tight closed container, away from light at 25 ˚c & 5 ˚c and compared with fresh samples, the Daclatasvir solution in 50% methanol was observed to be stable for this period.

LOD& LOQ: The limit of detection (LOD) and the limit of quantification (LOQ) were determined by injecting a series of samples of low concentration and from the calibration curve the LOD and LOQ were estimated as per ICH guidelines. LOD=3.3 σ/SLOPE and LOQ= 10 σ/ SLOPE. LOD and LOQ were found to be 0.03µg/ml and 0.08µg/ml respectively.

System suitability: The parameters of system suitability were calculated and the result was shown in table 10 below:

Table 10- System suitability

Parameter	Value
ƛ max	320 nm
Retention Time(min.)	2.33
Symmetry	0.88
Theoretical Plates	4943
Regression coefficient	0.9999

APPLICATION:

The developed validated method was applied for analysis of Andodaclta 60 mg tablets film coated tablet obtained from Al-andalous for pharmaceutical industries. The Daclatasvir eluted at 2.33 minute and no interfering peaks were founded .the recovery was calculated and the assay was 59.86 mg/ tab (99.77 %). So the method is applicable for the assay of Daclatasvir in tablets dosage form.

CONCLUSION:

Sensitive, simple, selective and accurate RP-HPLC-UV method was developed for analysis of antiviral drug (Daclatasvir) in tablet dosage form. The method is simple, rapid and helpful routine work for quick analysis of a large number of samples in short time.

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