Research Article

A Rapid Stability-indicating HPLC Method for Determination of Daclatasvir in Pure Form and Tablets

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ABSTRACT

A new, rapid, simple, precise and accurate stability-indicating reversed phase-HPLC method has been developed and validated for quantitative determination of daclatasvir (DAC) in pure form and tablets. An isocratic HPLC method, using Thermohypersile BDS C18 reversed phase column (150 mm × 4.6 mm i.d., particle size 5 µm) with isocratic ternary mobile phase consisting of methanol: acetonitrile: phosphate buffer (pH 3.0) (30:30:40, V:V:V), was investigated to separate DAC from its stress degradation products. The flow rate was 1.5 mL min-1 at ambient temperature and UV detector was used at 305 nm for detection. The elution time of DAC was found to be 2.195± 0.045 minutes. The developed method was validated for system suitability, linearity, accuracy, precision, limits of detection and quantitation, specificity, stability, robustness and for system suitability parameters as per ICH guidelines. The calibration curve was found to be linear with the equation y=51.399x + 4.8876, with a correlation coefficient of (R2=0.9999) over a concentration range of 2.0–120 µg mL-1. The limits of detection and quantification were 0.12 and 0.5 µg mL-1, respectively. The recovery value of this method is 99.0% and the reproducibility is within 0.90%. Stability tests were done through exposure of the analyte solution for five different stress conditions: Reflux with 1.0 mol L-1 hydrochloric acid (HCl), reflux with 1.0 mol L-1 sodium hydroxide (NaOH), reflux with 30% hydrogenperoxide (H2O2), exposure to ultraviolet radiation (UV) radiation and thermal conditions. The proposed method could be used for routine analysis of DAC in tablets.

Keywords: Daclatasvir, Stability indicating HPLC-method, C18 column, Method validation, stress degradation, Tablets.

INTRODUCTION

Daclatasvir dihydrochloride (DAC) is methyl [(2S)-1-{(2S)-2-[4-(4-{2-[(2S)-1-{(2S)-2-[(methoxy carbonyl)amino]-3-methyl butanoyl}-2-pyrrolidinyl]-1H-imidazol-4-yl}-4-biphenyl yl)-1H-imidazol-2-yl]-1-pyrrolidinyl}-3-methyl-1-oxo - 2-butanyl] carbamate dihydrochloride (Figure 1). DAC is an antiviral drug used to treat chronic (long-lasting) hepatitis C, a viral infection of the liver. DAC is an antiviral and acts directly against the hepatitis C virus [1]. DAC 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.

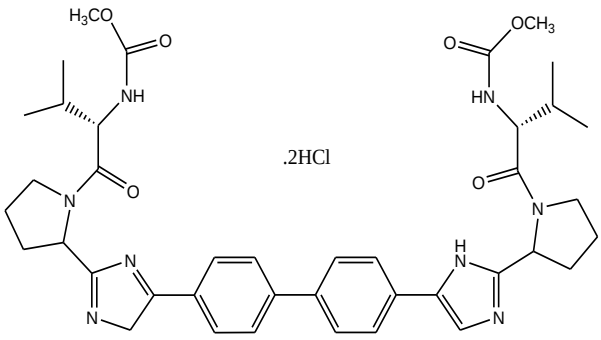

Figure 1: The chemical structure of daclatasvir dihydrochloride (DAC).

Literature survey reveals that there is few methods have been reported for the assay of DAC in pharmaceutical dosage forms, including high-performance liquid chromatography (HPLC) with ultraviolet or photodiode detection [2-4], upper high-performance chromatography (UHPC) [5], LC–MS/MS method [6-8], electrochemical method[9] and UV spectrophotometry [10, 11].

There is only few methods dealing with stability indicating methods for determination of DAC [2-5] but this method includes some drawbacks such as too long separation time and lower sensitivity. Also, there is no official pharmacopeial methods are available for the determination of DAC in drug substance and drug product. Therefore the aim of the proposed study is to find an inexpensive, new, sensitive, simple, accurate, precise, robust and rapid stability indicating RP-HPLC method applying isocratic mode for determination of DAC in bulk powder and tablets.

MATERIALS AND METHODS

Apparatus

Agilent HPLC series 1200 (Agilent technologies, Germany) consists of solvent pump (model G1311A), autosampler (model G1329A), column compartment (model G1316A) and UV detector (model G1314A). The pH measurements were made on compact inoLab pH Level 1 precision pH meter (Weilheim, Germany).

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Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from LAB-SCAN, Analytical Sciences (Gliwice, UL, Sowinskiego, Poland). NaOH, HCl and 30% H_2O_2 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Potassium dihydrogen orthophosphate obtained from Lobachemie, India. DAC raw material was obtained from Adwia Pharmaceutical Co. (El-Obour City, Cairo, Egypt). Bi-distilled water was used throughout the work.

Pharmaceutical dosage forms

Daklinza 30 mg film-coated tablets contain 30 mg DAC per tablet and were produced by Pharmaceutical Co. (El-Obour City,Cairo, Egypt). Daclahepex 60 mg tablets contain Daclatasvir dihydrochloride 66 mg equivalent to 60 mg DAC were produced by Global Napi Pharmaceuticals, 6th October City, Giza, Egypt.

Chromatographic conditions

The chromatographic separation was performed using BDS- Thermohyersil C18 (150 mm × 4.6 mm), 5.0 µm particle size column; the column temperature was maintained at 25 ± 2 ºC. The Autosampler utilized methanol as a rinse solution, the total run time was 5.0 minutes. The elution quaternary pump ran an isocratic flow using mobile phase consisting of a mixture of methanol, acetonitrile and phosphate buffer (pH 3.0) (30:30:40, V:V:V) at a flow rate of 1.5 mL min^{-1}. The eluate was monitored at 305 nm using UV-detector. The retention time of the drug was found to be 2.195±0.045 min. The injection volume was 20 µL.

Preparation of stock and standard working solutions

A stock solution of DAC (1000 µg mL^{-1}) was prepared by dissolving 100 mg of DAC in acetonitrile and water (1:1 v/v) in 100 mL volumetric flask, then shake and sonicate for 10 min till completely dissolved and then, complete the volume to 100 mL with the same solvent. The working standard solutions were prepared by diluting aliquots of stock solution with acetonitrile and water (1:1 v/v) to obtain final concentrations ranging from 2.0 to 120 µg mL^{-1}. Working solution of the drug was stable for one week.

Construction of calibration curve

Aliquots of standard solution, ranging from 2.0 to 120 µg mL^{-1} were prepared in a series of 10 mL volumetric flasks. 20 µL was injected into the instrument. Detection was performed at wavelength 305 nm. The calibration graph was constructed by plotting the peak areas obtained at the wavelength 305 nm versus the corresponding injected concentrations.

Procedure for dosage forms

Ten tablets (Daklinza 30 mg or Daclahepex 60 mg film-coated tablets) were weighed, finely powdered and an accurately weighed amount of the powdered tablets equivalent to 30 mg of DAC was dissolved in 50 mL of the mobile phase methanol, acetonitrile and phosphate buffer (pH 3.0) (30:30:40, V:V:V) in 100 mL volumetric flask. The flask sonicated for 10 min and the solution was filtered through a 0.45 µm membrane filter and then the final solution was completed to the mark with the mobile phase. The procedure was then completed as mentioned above under the general procedure.

Stability tests

Forced degradation studies were performed to provide an indication of the stability-indicating properties and specificity of the method. Intentional degradation was attempted using acid hydrolysis, base hydrolysis, hydrogen peroxide oxidative degradation, and thermal degradation and UV-radiation degradation. A degradation sample was prepared by dissolving 10 mg of DAC in 100 mL acetonitrile and water (1:1 v/v) through shaking and sonication. Then 10 mL of this solution was taken in each of three 50 mL round bottomed flasks to perform the first three degradation tests. To the first flask, 10 mL of 1.0 mol L^{-1} HCl was added for acidic degradation. To the second flask, 10 mL of 1.0 mol L^{-1} NaOH was added for basic degradation. To the third flask, and 10 mL of 30 %(v/v) H_2O_2 was added for oxidative degradation. All the three flasks were refluxed for about 1.0 h at 60 °C. After completing degradation treatments, samples were allowed to cool to room temperature and treated as follows: The pH values of the first and second flasks were neutralized with 1.0 mol L^{-1} NaOH and 1.0 mol L^{-1} HCl, respectively. To the third flask 1.0 N sodium bisulfite solution was added to destroy H_2O_2. The volume of all the three flasks was adjusted to 50 mL with the mobil phase. Suitable aliquots of resultant degradation samples were taken and subjected to analysis after suitable dilutions with the mobil phase against the control samples (which lacked the degradation treatment) and 20 µL solution was injected into the system and the chromatogram was recorded to assess the stability of samples.

For thermal degradation, DAC powder was dispersed onto a Petri-dish and left in an oven at 60 °C for 24 h then the solution was prepared from it in a concentration of 100 µg mL^{-1} using the mobile phase as solvent.

For degradation through UV-radiation 2.0 mL of the sample was retained in the UV radiation from 5.0 to 60 minutes and then the radiated solution diluted with methanol to 10 mL, then finally injected into the LC and compared with the control sample.

Method validation

The methods were validated according to the International Conference on Harmonization Guidelines [12] for validation of analytical procedures.

Linearity

Linearity of the method was tested for different concentrations in a range from 2.0 to 120 µg mL^{-1}. A graph was plotted between the peak areas versus concentrations to obtain the calibration curve. The seven

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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.

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.

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 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.

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. Accuracy of the method was tested by % recovery of DAC on three concentrations; each concentration was repeated three times. Known amounts of DAC were added to a synthetic mixture of the drug product components (placebo) and subjected to analysis procedure. 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. Robustness was examined by small change in the temperature (±2.0 °C), flow rate (±0.1 mL/min), percentage of methanol and acetonitrile solvents solvent (±1%), wavelength of detection (±2.0 nm), and injection volume (±1.0 µL).

Solution stability

Sample solution and the standard solutions containing DAC 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 2-5°C. Freshly prepared solutions and the solutions which were stored at room temperature and refrigerated condition (2-5°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

System suitability

The conditions affecting the chromatographic performance of DAC were carefully studied in order to recognize the most suitable chromatographic system. So, the optimum chromatographic performances were achieved via using isocratic mobile phase composed of methanol, acetonitrile and phosphate buffer (pH 3.0) (30:30:40, V:V:V), injection volume 20 µL, column temperature 25°C, detection wavelength 305 nm and flow rate 1.5 mL min^{-1}. The results of three runs indicate high system suitability (Table 1).The retention time (t_R) value of DAC was 2.195±0.045 minutes. The RSD of peak area was 0.90%.

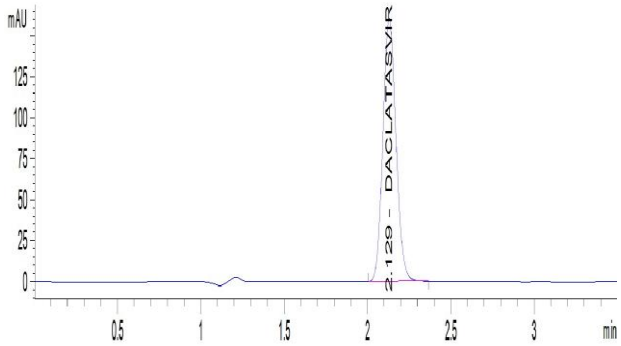
Table 1: System suitability and regression data.

Parameters	Results
System suitability	
t_R± SD (min)	2.195 ± 0.045
N	10752
k'	5.426
Linearity and regression data	
Linearity range (µgmL^{-1})	2.0-120
Detection limit (µgmL^{-1})	0.12
Quantitation limit (µgmL^{-1})	0.5
Slope (b)± RSD	51.399 ± 0.423
Intercept (a) ±RSD	4.8876 ± 0.04
Coefficient of determination (R^2)	0.9999

[a]Theoretical values for *t* and *f*at confidence limit at 95% confidence level and five degrees of freedom (p= 0.05) are 2.179 and 3.84, respectively.

Selectivity, specificity and stability of the method

The resulted peak after tablet analysis is found to be homogeneous and there are no co-eluting peaks indicating specificity of the method. Comparison between the chromatogram of the raw DAC and that of extracted DAC from tablets indicates that the excipients in the formulation did not interfere with the determination of (Figure 2).



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(b)

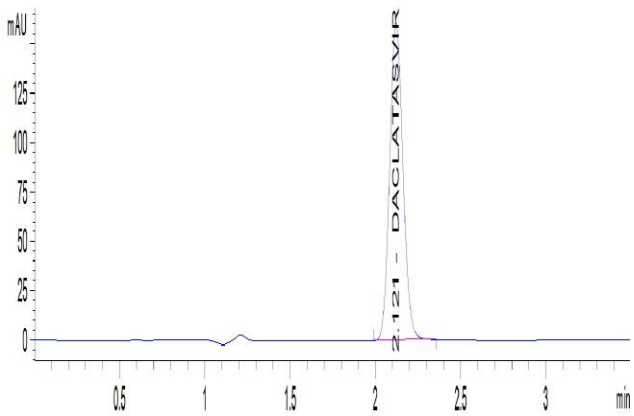

Figure 2: Chromatograms of (60 µgmL^{-1}) DAC from (a) raw material and (b) tablets.

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, degradents, matrix (placebo) etc. Specificity was tested by injecting placebo preparation and forced degradation samples. Forced degradation studies were performed to provide an indication of the stability indicating property and specificity of the proposed method. Forced degradation was attempted to stress conditions like acid hydrolysis, base hydrolysis, peroxide oxidation, thermal degradation and photolytic degradation. To check and ensure the homogeneity (peak purity) of peak in the stressed sample solutions, photo diode array detector was employed. In forced degradation study it was observed peak purity in all the degradation conditions has been proven for DAC peak. Results are tabulated in Table 2.

Table 2: Forced degradation data of DAC.

Degradation conditions	%Assay observed
Acid	98.5
Base	99.2
Oxidative (H_2O_2)	98.1
Thermal	99.4
UV	98.0

Stability of the standard solution was studied by injection of the prepared solution at periodic intervals into the chromatographic system up to about 5.0 days. The results indicate that the RSD of the peak area was within 0.87%.

Linearity, LOD and LOQ

Different concentrations of DAC solution ranging from 2.0–120 µg mL^{-1} were analyzed. The graph of the peak area against concentration proved linear in the range of 2.0–120 µg mL^{-1} and the linearity equation is: y = 51.399x + 4.8876 and the regression coefficient= 0.9999 (Figure 3). The results have indicated good linearity. The limit of detection (LOD) is defined as the injected quantity giving S/N of 3.0 (in terms of peak height) and was found to be 0.12 µg mL^{-1}. The limit of quantification (LOQ) is defined as the injected quantity giving S/N of 10 (in terms of peak height) and was found to be 0.5 µg mL^{-1} (Table 1).

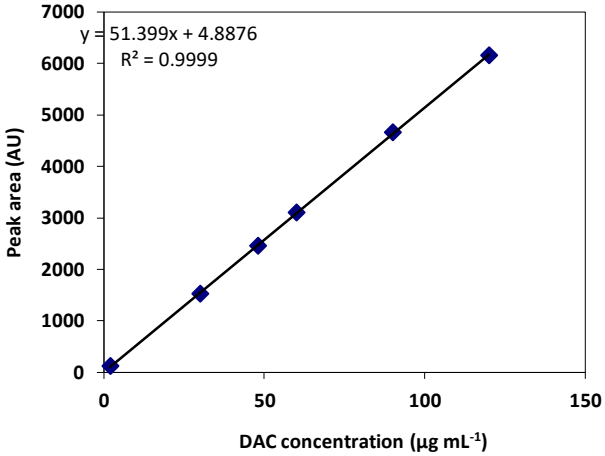

Figure 3: Linearity graph of DAC.

Reproducibility, precision and accuracy of the method

Intra-day precision was assessed by injection of the standard solution at three concentrations five times during a day. The same was done for inter-day precision test except that the injection of the samples were every day for five days. Results (Table 3) show that there were high intra- and inter-day precisions. The RSD was calculated for results (%RSD ≤ 0.84%) and it indicates that proposed method has got acceptable level of repeatability.

Table 3: Reproducibility, precision and accuracy (n=5).

Injected amount (µg mL^{-1})	Intra-day (n=5)			Inter-day (n=5)		
	Observed amount ± S.D.	Precision (RSD%)*	Accuracy (%)**	Observed amount ± S.D.	Precision (RSD%)*	Accuracy (%)**
30	29.75 ± 0.18	0.60	99.20	29.55 ± 0.12	0.40	98.50
60	59.40 ± 0.50	0.84	99.0	60.30 ± 0.44	0.73	100.50
90	90.20 ± 0.38	0.42	100.20	89.46 ± 0.57	0.64	99.40

*RSD (%) = S.D. x 100/mean

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Accuracy of an analytical method is the closeness of the test results obtained by the method to that of true value. Accuracy of the proposed method was established by recovery experiments. This study was conducted by preparing and analyzing samples at 50%, 100% and 150% of targeted concentration (60 µg mL^{-1}), in triplicate and injected into the chromatographic system. Results obtained from recovery studies are given in Table 4.

Table 4: Statistical analysis of results obtained by the proposed method applied on tablets compared with a reported method.

	Proposed method[a]		Reported method [4]
	Daklinza tablets	Daclahepex tablets	
N	5	5	5
Mean recovery	99.20	99.60	100.10
± SD	0.71	0.52	± 0.73
± R.S.D%	0.72	0.52	± 0.73
Variance	0.50	0.27	0.53
S.E	0.32	0.23	0.33
t-value [b]	1.76	1.12	
F-value [b]	1.06	1.97	

[a]Average of five determinations (n = 5).

[b]Theoretical values for *t* and *f*at confidence limit at 95% confidence level and five degrees of freedom (p= 0.05) are 2.776 and 6.39, respectively.

Application

Analysis of DAC in Daklinza 30 mg or Daclahepex 60 mg tablets by the proposed method showed high accuracy with mean recoveries of 99.0±0.52% and 99.20 ±0.71%, respectively (Table 4). The results were compared with a reported method[4]. The calculated values of *f* and *t* indicate that there is no significant difference between both methods.

Robustness of the method

The robustness of the present method was evaluated within small variation in its parameter and was found to be robust. Robustness was examined by small change in the temperature (±2.0 °C), flow rate (±0.1 mL/min), percentage of methanol and acetonitrile solvents (±1%), wavelength of detection (±2.0 nm), and injection volume (±1.0 µL) (Table 5). The slight variations in the examined factors had no significant effect on the peak areas or retention times (t_R-values).

Table 5: Robustness of the proposed method.

Changes factors	Temp. (°C)		Flow rate (mL/min)		Mobile phase (%)				Wavelength of detection (nm)		Injected volume (µL)	
					Methanol		Acetonitrile					
Changes	23, 25 and 27		1.40, 1.50 and 1.60		29, 30 and 31		29, 30 and 31		303, 305 and 307		19.0, 20 and 21	
Tested parameter	Peak area	t_R	Peak area	t_R	Peak area	t_R	Peak area	t_R	Peak area	t_R	Peak area	t_R
C.V. (%)	1.75	0.05	2.30	0.08	2.45	0.10	1.90	0.07	2.10	0.12	1.90	0.13

CONCLUSIONS

A valid and rapid stability-indicating HPLC-method for the quantification of DAC in pure form and tablets was established. Compared with the published chromatographic methods, this method represents a strong reduction of the analysis time and it is considered as a stability indicating method. The full run time for separation of the intact DAC from its degradants is about 5.0 minutes which is very short comparing with the previously published work. With the proposed method a satisfactory separation of DAC from the degradation products, extended linear range, and rapid analysis time were carried out. A high recovery of DAC in tablets was achieved. The proposed method ensured a precise and accurate determination of DAC in tablet formulations and is a stability-indicating method.

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