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RP-HPLC method development and validation for quantification of daclatasvir dihydrochloride and its application to pharmaceutical dosage form

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Abstract: Daclatasvir dihydrochloride is an antiviral drug used in the treatment of Hepatitis C and for its estimation in drug product, no Pharmacopeial method is available. Therefore, a simple, rapid, precise and accurate isocratic RP-HPLC method was developed and validated for quantification of daclatasvir dihydrochloride in pharmaceutical dosage form. The quantification was carried out using Hypersil ODS - C18 Column (250mm, 4.6mm, 5µm), Shimadzu LC-2030 Prominence-I Series. The mobile phase composed of phosphate buffer (pH 3.5, adjusted with ortho phosphoric acid) and acetonitrile (60:40 v/v). The flow rate was 1.0ml/min with UV detection at 308 nm. The validation of developed method was conducted for specificity, linearity, accuracy, precision, LOD and LOQ. A linearity was established in the concentration range of 0.5-150% with coefficient of correlation 0.9993. The limit of detection (LOD) was 0.005µg/ml and the limit of quantification (LOQ) was 0.01µg/ml. The method was successfully applied to the assay and *in-vitro* dissolution studies of daclatasvir dihydrochloride in tablet dosage form. It can be concluded that this method can be very helpful in the quality control estimation of daclatasvir dihydrochloride in different pharmaceutical products intended for hepatitis C infections.

Keywords: RP-HPLC, daclatasvir dihydrochloride, method validation, dosage form.

INTRODUCTION

Daclatasvir is classified in antiviral drug acting as HCV nonstructural protein 5A (NS5A) inhibitor. The chemical name of daclatasvir dihydrochloride is carbamic acid, N,N′-[[1,1′-biphenyl]-4,4′-diylbis[1Himidazole-5,2-diyl-(2S)-2,1-pyrrolidinediyl[(1S)-1-(1-methylethyl)-2-oxo-2,1-ethanediyl]]]bis-, C,C′-dimethyl ester, hydrochloride (1:2) as shown in fig. 1 (Lee, 2013, Gentile *et al.*, 2014). Daclatasvir is an inhibitor of NS5A, a nonstructural protein encoded by HCV. It binds with the N-terminus within Domain 1 of the protein, thus, distort the structure which restrict the functions of NS5A and inhibits both replication of viral RNA and virion assembly. Daclatasvir is used as HCV NS5A virus inhibitor with sofosbuvir, with or without ribavirin, for the treatment of chronic HCV genotype 1 or 3 infection (Lee, 2013; McCormack, 2015). The daily recommended dose of Daclatasvir is 60 mg, which can be administered orally with or without food. The recommended dose, treatment and duration of daclatasvir with sofosbuvir & ribavirin is not yet established for patients of HCV genotype 1 with Child-Pugh C cirrhosis or patients of HCV genotype 3 with cirrhosis (Bakshi and Singh, 2002; Chakravarthy and Sailaja, 2016a; Gentile *et al.*, 2014). Validation is a process of collection of documentary evidence that any of the procedure, process, method, or activity being adapted is capable of producing consistent and satisfactory result in terms of measurements or in terms of product quality. To demonstrate that a pharmaceutical product manufactured with any process in any pharmaceutical company, it is required to validate many procedures, processes, methods activities associated with pharmaceutical manufacturing including machinery, skills, testing procedures and methods (Shabir, 2003; FDA, 2008). The literature survey showed that several methods have been reported for the estimation of daclatasvir in combination with Sofosbuvir in solid dosage form (Eldin *et al.*, 2017) and individual daclatasvir by using HPLC (McCormack, 2015; Chakravarthy and Sailaja, 2016a). In the present investigation, a simple, economical, precise, accurate RP-HPLC method has been developed and validated for the quantitative determination of daclatasvir dihydrochloride from the tablet dosage form.

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MATERIALS AND METHODS

Chemicals and reagents

All other chemicals and reagents used in the study were of HPLC grade quality. Daclatasvir HCl (working standard) was taken as gift sample from PharmEvo Ltd. HPLC grade potassium dihydrogen phosphate, 1-Heptane sulphonic acid, acetonitrile and phosphoric acid were purchased from Merck KGaA, Darmstadt, Germany. Highly purified distilled water collected from plant of PharmEvo private limited. Brand of daclatasvir tablets were purchased from pharmacy.

Instrumentation

The HPLC system (Shimadzu LC-2030 Prominence-I Series), consisted of LC-20AT VP pump, Hypersil ODS C18-Column (250mm x 4.6mm, 5µm) and LC Solution software, was used in the study. In additions, analytical Balance (Mettler Toledo Germany), Dissolution apparatus, (Pharmatest Germany), Disintegration apparatus (Pharmatest Germany), Sonicator (Elma Germany), PH Meter (WTW Ino Lab-Germany), Filtration assembly (Sartorius, Gorringen, Germany), Vacuum pump (China) and Varnier caliper (China) were also used in the study.

Chromatographic conditions

An isocratic HPLC system equipped with Schimadzu UV detector, LC-20AT VP pump and Hypersil ODS C18 (250 mm × 4.6 mm, 5µm) column was used for separation and the chromatograph were recorded using LC Solution software. The flow rate was 1.0ml/min with UV-Vis detection at 308nm. Injection volume, run time and retention time were 10µL, 6min and 3.895min., respectively. fig. 2 show the chromatogram of daclatasvir dihydrochloride.

Mobile phase preparation

Accurately weighed amount (6.8g) of potassium dihydrogen phosphate was transferred to 1000mL volumetric flask and dissolved in distilled water. The pH of the phosphate buffer was adjusted at 3.5 with ortho-phosphoric acid (OPA). The mobile phase composed of phosphate buffer and acetonitrile in the ratio of 60:40 (v/v).

Preparation of stock and working standard solution

For the preparation of standard stock solution, approximately 55mg of daclatasvir dihydrochloride (equivalent to 50.06mg daclatasvir) was accurately weighed and dissolved with HPLC grade water into 100 mL volumetric flask to obtain 0.501mg/mL. Working standard solutions of daclatasvir dihydrochloride were prepared in mobile phase to produce final concentrations of 2.497, 4.993, 24.698, 49.396, 156.269, 259.551, 310.743, 414.922, 450.846, 499.344, 543.351, 583.765, 616.097 and 676.269µg/mL.

Preparation of samples

Commercially available brand, containing daclatasvir dihydrochloride was used for preparation of samples. Twenty tablets were accurately weighed and triturated to obtain a homogeneous mixture. Powder equivalent to the average weight of twenty tablets (equivalent to 50.06mg of Daclatasvir dihydrochloride) was transferred to a 100 ml volumetric flask, 25ml of diluent was added to dissolve & sonicated for 25minutes, then mixed magnetically for 30 minutes and made up volume with diluent to obtain a concentration of 501µg/ml. Filtered this solution through 0.45µ before injecting of 10µL.

Stability of samples solution

For quantitative analysis, stability of sample is an important requirement. The stability of samples solution was carried out by keeping the samples at room temperature for 12h. Similarly, evaluation was also conducted at -15 to -20°C for 7 days (Fayyaz *et al.*, 2015).

Selection of wavelength

To obtain the wavelength at which maximum absorbance achieved, spectrophotometer was applied for scanning of the daclatasvir dihydrochloride standards in the UV region (200-400 nm). The final detection of the drug was then carried out at 308 nm. After successful scanning, different samples of tablets were also run on HPLC at different wavelength and 308 nm wavelength was found satisfactory for daclatasvir dihydrochloride. The peak of daclatasvir dihydrochloride was detected at 3.895 min.

Method validation

The developed method of analysis was validated as per International Council for Harmonisation (ICH) guideline for the parameters such as system suitability, specificity, linearity, accuracy and precision, limit of detection (LOD) and limit of quantification (LOQ).

System suitability

System suitability tests are an integral part of chromatogramic method, used to verify reproducibility of the chromatogramic system. Initially, the HPLC system was stabilized for 40 minutes. The system suitability parameters were determined by repetitively injecting six replicates of drug solution at a concentration of 501µg/mL to check the reproducibility of the system. To ascertain the system suitability of the proposed method, the parameters like, peak area, RSD, retention time and tailing factor were recorded.

Specificity

The effect of excipients and other additives usually present in the tablet formulation of daclatasvir dihydrochloride was investigated. In order to determine the specificity of the analytical method, the most commonly used excipients such as lactose anhydrous, microcrystalline cellulose and magnesium stearate have been incorporated in the solution containing of daclatasvir dihydrochloride (501µg/mL) was injected (triplicate) and tested. The representative chromatogram showed no additional peak other than drug was observed. The mean % recovery for daclatasvir dihydrochloride was obtained as 100.014% that also verified that this method could be used to quantify the daclatasvir dihydrochloride in pharmaceutical dosage forms (Fayyaz *et al.*, 2015).

Linearity

In order to determine the linearity and to construct a calibration curve, fourteen concentrations (0.5% to 120%) i.e. 2.497, 4.993, 24.698, 49.396, 156.269, 259.551, 310.743, 414.922, 450.846, 499.344, 543.351, 583.765, 616.097 and 676.269µg/mL, were injected (Dong, 2006a).

Method of least square analysis was applied for the determination of coefficient of correlation value. A calibration curve was plotted between concentration *versus* area response and statistical analysis of the calibration curve is shown in fig. 3.

Accuracy (Recovery studies) and precision

The accuracy of an analytical procedure expresses the degree of closeness of agreement between the values which is accepted either as a conventional true value or an accepted reference value and the value found (Horowitz and Latimer, 2006; ICH, 2002). Accuracy of the method was determined by recovery study of daclatasvir dihydrochloride, in which three different known concentration levels (80%, 100% and 110%) of daclatasvir dihydrochloride solutions were added to the samples containing known contents and the percentage of recovery was calculated by comparing the determined amount of these standards with the amount added to the samples. The mean percentage recovery of daclatasvir dihydrochloride at each level was observed in the range of 100.014-103.354% and results are shown in table 2 (Jamil S *et al.*, 2005).

The method was also validated for intra- and inter-day precisions. Precision of the anticipated method was checked by repeatability (inter-day precision) and intermediate precision (intra-day precision) at six different weight levels ranges from 223.6-226.3mg. Intraday precision was performed on the same day, whereas, interday precision was performed on the different days. The percentage relative standard deviation (RSD) of both intra as well as interday was calculated and results are given in table 3 and 4, respectively. The calculated RSD should be less than 2%.

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

Limit of detection is the lowest concentration in a sample that can be detected, but not quantified under the same experimental condition, whereas, limit of quantification (LOQ) is the lowest concentration of drug in the sample that can be quantified with acceptable accuracy and precision. LOD and LOQ were determined by analyzing five samples of different lower concentrations i.e. 0.08, 0.04, 0.02, 0.01 and 0.005µg/mL of daclatasvir dihydrochloride. LOQ and LOD were calculated by using the following equations (Ravisankar *et al.*, 2013).

$$LOD = 3.3 \times SD/S \quad (1)$$

$$LOQ = 10 \times SD/S \quad (2)$$

Where, SD is the standard deviation of the peak areas of the drugs, taken as a measure of noise and S is the slope of the corresponding calibration curve.

Determination of daclatasvir dihydrochloride in the marketed brand using this method

The potency of the daclatasvir dihydrochloride present in the marketed brand was also determined by using the current developed and validated method and the result is given table 7.

RESULTS

Linearity, system suitability and precision

The method displayed good linearity correlation for daclatasvir dihydrochloride and it was obtained as 0.9996 (fig. 3). System was evaluated by injecting 6 runs of standard samples on each day of method validation. System suitability parameters tests were, %RSD (Relative Standard Deviation) of retention time, peak area and tailing factor (Lister, 2005, Dong, 2006b), which were within the range (see in table 1). The method was also validated for intra-day and inter-day accuracy and precision. The intraday accuracy was found in the range of 97.87-99.24% while interday accuracy was observed in the range of 98.85-99.88%, as shown in table 3 and 4.

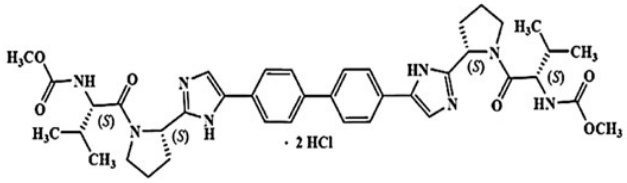

Fig. 1: Chemical structure of daclatasvir dihydrochloride

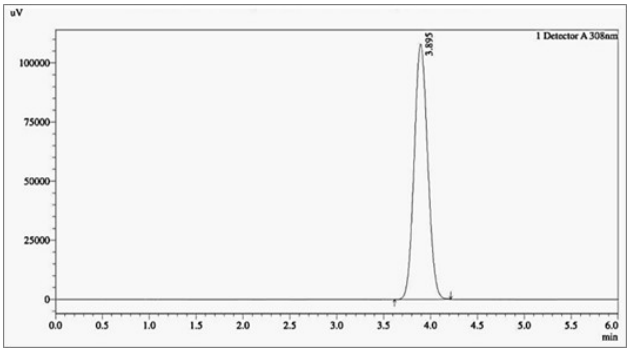

Fig. 2: Chromatogram of daclatasvir dihydrochloride

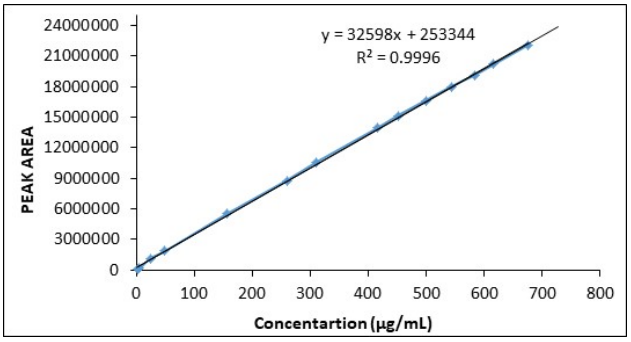

Fig. 3: Linearity curve of daclatasvir dihydrochloride in different concentration (µg/mL)

Analytical recovery study

Percentage recovery tests were conducted by adding known amounts of standard solutions to sample followed by analysis using proposed method. Three runs were performed for every concentration and then peak area was calculated as shown in table 2.

Table 1: System suitability peak area of daclatasvir dihydrochloride

No of Runs	Peak Area	Mean Peak Area	Standard Deviation (SD)	% RSD	Mean Retention Time (min.)	Tailing Factor
Run 1	16518984	16509939	6695.363	0.041	3.896	1.2
Run 2	16515884					
Run 3	16508327					
Run 4	16509016					
Run 5	16507315					
Run 6	16500108					

Table 2: Percentage recovery for accuracy of daclatasvir dihydrochloride

No. of Sample	Amount/ conc. of drug	Peak Area (Run A)	Peak Area (Run B)	Peak Area (Run C)	Mean Peak Area	% drug found	% Recovered
1	80%	14003839	14010259	14008539	14009399	81.9	102.380
2	100%	16755839	16758031	16756900	16757465.5	100.00	100.014
3	110%	19059351	19059682	19054570	19057126	113.7	103.354
Mean					101.916%		
Standard Deviation (SD)					1.717		
Relative standard deviation (RSD) (Limit: NMT 2.0%)					1.685%		

Table 3: Intraday precision characteristics of daclatasvir dihydrochloride

Weight of Sample (mg)	Injection Volume (µL)	Mean Peak Area	Theoretical Percentage (%)	Percentage Found (%)
223.6	10	16582441	100	99.24
224.0	10	16567700	100	98.97
225.5	10	16573250	100	98.35
226.3	10	16550634	100	97.87
225.1	10	16606547	100	98.72
225.3	10	16576508	100	98.46
Mean			98.60%	
Standard Deviation (SD)			0.49	
Relative standard deviation (RSD) (Limit: NMT 2.0%)			0.49%	

Table 4: Interday precision characteristics of daclatasvir dihydrochloride

Weight of Sample (mg)	Injection Volume (µL)	Mean Peak Area	Theoretical Percentage (%)	Percentage Found (%)
225.5	10	16564614	100	99.42
225.0	10	16571545	100	99.68
224.6	10	16574772	100	99.88
225.0	10	16566149	100	99.65
226.1	10	16513139	100	98.85
225.6	10	16571277	100	99.42
Mean			99.48%	
Standard Deviation (SD)			0.36	
Relative standard deviation (RSD) (Limit: NMT 2.0%)			0.36%	

Table 5: Limits of detection and limit of quantification for daclatasvir dihydrochloride

Concentration (µg/ml)	Back Calculated Concentration (µg/ml)					Mean	SD	% Assay	Peak Area
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5				
0.08	0.078	0.079	0.077	0.081	0.081	0.078	0.002	97.50	6678.00
0.04	0.039	0.038	0.038	0.042	0.041	0.038	0.002	95.83	3415.00
0.02	0.018	0.021	0.019	0.024	0.017	0.019	0.003	96.67	1953.00
0.01	0.011	0.008	0.01	0.009	0.008	0.010	0.001	96.67	1495.00
0.005	0.0045	0.0047	0.0048	0.0047	0.0046	0.005	0.000	93.33	1187.00
0.0025	0.0022	0.0028	0.0022	0.0026	0.0024	0.0024	0.000	0.00	Not detected

Table 1: Stability of standard solutions

Drug	Condition	Theoretical Conc. (µg/mL)	Obtained Results (µg/mL)	% Recovery	% RSD
Daclatasvir dihydrochloride	Room temperature (12 h)	90	88.973	98.859	0.741
Daclatasvir dihydrochloride	-15 to -20^{o}C (7 days)	90	86.564	96.183	0.723

Table 7: Determination of drug content present in marketed brand of daclatasvir dihydrochloride by this proposed method

Analyte	Measured Amount Mean ± SD	Claimed Amount (mg)	% potency*
Daclatasvir dihydrochloride	29.742±0.232	30	99.14
*Potency = Measured amount × 100/Claimed amount			

Limit of detection (LOD), limit of quantification (LOQ) and Solution stability

The limit of detection (LOD) and limit of quantification (LOQ) were calculated by using Eqs. [1] and [2], respectively. LOD and LOQ for daclatasvir dihydrochloride were 0.005 and 0.01µg/mL, respectively and result are given int 5. The stability of standard solutions was also performed, and the results are mentioned in table 6.

Assay of marketed tablets brand

The assay of marketed tablet brand of daclatasvir dihydrochloride was also performed by using the current developed and validated method and the potency was found 99.14%, as given in table 7.

DISCUSSION

The aim of the study was to develop a simple, isocratic, accurate and sensitive RP-HPLC method for the determination of daclatasvir dihydrochloride in pharmaceutical solid dosage form. Hypersil ODS C18 (250mm × 4.6mm, 5µm) column was used for separation, which provided the best peak shapes and efficiencies. The method was validated as per International Council for Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH, 2002). All the parameters such as specificity, linearity, accuracy, precision, and percent recovery were found within the acceptable limits. The best resolution, separation and no interference from the mobile phase appeared at 308 nm. The injection volume, run time and retention time were 10µL, 6min and 3.895min. respectively.

Linear regression by the least square method was used for the determination of coefficient of correlation for linearity, which was 0.9996, indicating excellent linearity (fig. 3). System suitability was evaluated and tests parameters were, %RSD of retention time, peak area and tailing factor (Lister, 2005, Dong, 2006b), which were found within the acceptance range (see in table 1). Previously a method reported having calculated linear regression coefficient value of 0.9992 (Chakravarthy and Sailaja, 2016b). The method was also validated for intra-day (97.87-99.24%) and inter-day (98.85-99.88%) accuracy and precision. The precision is one the most important step of the method validation, for which repeatability test was performed by preparing six different samples solutions. Precision of the anticipated method was determined by repeatability (inter-day precision) and intermediate precision (intra-day precision), which was expressed as relative standard deviation (RSD) (table 3 and 4).

The average %age recovery for each level was calculated as per Association of Official Analytical Chemists International (Lister, 2005; Association of Official Analytical Chemists and Association of Official Agricultural Chemists (US), 1920; ICH, 2002). It was concluded that the excipients used in the formulation did not interfere with drug present in tablets and selected medium did not absorb the drug to any extent. The obtained percentage recovery indicated that the method has a high degree of accuracy for determination of daclatasvir dihydrochloride (table 2).

The calculated LOD (0.005µg/mL) and LOQ (0.01 µg/mL) for daclatasvir dihydrochloride, indicating that the method could be very useful for any country following relevant quality standards (table 5). An important validation parameter is the stability of standard solutions, which was performed too (table 6). The assay of marketed tablet brand of daclatasvir dihydrochloride was found 99.14% when used the current developed and validated method as shown in table 7.

CONCLUSION

It was concluded that during the present study, a simple, accurate, rapid, precise, reproducible and inexpensive RP-HPLC method was developed with excellent correlation co-efficient for the estimation of daclatasvir dihydrochloride in pharmaceutical solid dosage forms. The developed RP-HPLC method was validated according to ICH guidelines for validation of analytical procedure. Moreover, the mobile phase is cheap and easily available. The shorter retention time made the

method easier, less time consuming for analysis and applicable pharmaceutical industry especially in quality control department.

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