A STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF DABIGATRAN IN PURE AND PHARMACEUTICAL DOSAGE FORMS

Mr. BRC Sekhar Reddy, Dr. Nallagatla. Vijaya Bhaskar Rao

Research Scholar, Department of Chemistry, Acharya Nagarjuna University, Andhra Pradesh, India.

Principal, Govt. Degree College, Alair, Nalgonda Dist, Andhra Pradesh, India.

ABSTRACT

A validated reversed phase high performance liquid chromatography is used for the estimation of Dabigatran in bulk and pharmaceutical dosage form. It is a simple, precise, accurate reversed phase liquid chromatography. The HPLC method has been carried out by using Chromasil C18 column. This method has been developed by using the mobile phase consisting methanol: acetonitrile: water 80:15:5(v/v). (pH 4.7) at the flow rate of 1.0ml/min by the detection of UV at 280nm. The retention time of the dabigatrin is 5.73min. The runtime is 10min. the linearity was found to be over a concentration of 40-100µg/ml respectively. The accuracy was found to be 98.62-99.81%. With a correlation coefficient of 0.999.The proposed method can be used for the estimation of the drug in bulk and pharmaceutical formulation. The results of analysis have been validated satisfactorily using recovery studies.

Keywords: RP-HPLC, Dabigatran.

1. INTRODUCTION

Dabigatran is an oral anticoagulant from the class of the direct thrombin inhibitors. Dabigatrin is chemically 3-({2-[(4-Carbamimidoyl-phenylamino)-methyl]-1-methyl-1*H*-benzoimidazole-5-carbonyl}-pyridin-2-yl-amino)-propionic acid[1]. Dabigatran is an anticoagulant which prevents blood clots. It is an anticoagulant medicine used for the prevention of clots and emboli after orthopedic surgery (hip or knee replacement) and to prevent stroke and other systemic emboli in people with non-valvular atrial fibrillation (AF), a commonly occurring abnormal heart rhythm[2,3]. Dabigatran is generally ingested orally and is administered in capsule form [4].

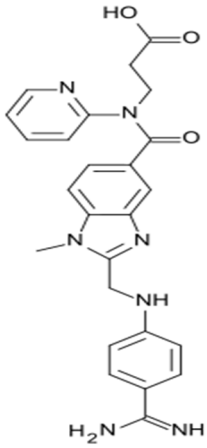


Figure 1: Molecular structure of Dabigatran

Thrombin plays a role in the last step of blood coagulation, being composed of one active site and two secondary binding exosites. The first exosite aids active site binding through docking substrates such as fibrin, whilst the second binds heparin. Dabigatran thus inactivates both fibrin-bound and free thrombin through binding to the active site; proving more effective than indirect thrombin inhibitors such as unfractionated heparin (which cannot inhibit fibrin-bound thrombin)[5]. Very few HPLC methods have been found in literature so as to estimate the Dabigatran in pure and pharmaceutical dosage form. The present study deals with the estimation of Dabigatran in pure drug and tablet dosage form available in the market.

2. Materials and methods

2.1Materials

Dabigatran standard was obtained from Hetero labs, Hyderabad and formulation tablets were purchased from local pharmacy store. HPLC grade Methanol, Water, Acetonitrile and analytical grade Chemical reagents were purchased from Merck specialties Pvt.limited, Mumbai. **2.2 Instrumentation:**

Chromatographic separation was performed on a PEAK chromatographic system equipped with LC-P7000 isocratic pump; Rheodyne injector with 20µl fixed volume loop, variable wavelength programmable UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Teccomp UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Sonicator (1.5L) Ultrasonicator was used to sonicating the mobile phase and samples. Standard and sample drugs were weighed by using Denver electronic analytical balance (SI-234) and pH of the mobile phase was adjusted by using Systronics digital pH meter.

2.3Determination of maximum absorbance

The standard solutions of Dabigatran were scanned in the range of 200-400 nm against mobile phase as blank. Dabigatran showed maximum absorbance at 280nm.Thus the wave length selected for the determination of Dabigatran is 280 nm.

2.4 Preparation of standard stock solutions

Accurately weighed 10mg of Dabigatran is dissolved in 10ml of Methanol which is considered as stock solution. Working standard solutions were diluted further to get concentration range 40-100µg/ml. Each of these drug solution (20µl) was injected into the column, the peak area and retention time were recorded.

2.5 Preparation of tablet formulation

10 tablets of Dabigatran were weighed, powdered and an amount of tablet powder equivalent to 10 mg of Dabigatran was accurately weighed and transferred in a 10ml of methanol. This mixture was subjected to sonication for 10 min and filtered through a 0.45µm nylon membrane filter paper. The final sample solution was diluted properly to prepare a concentration of 1000µg/ml of Dabigatran. The resultant solution used for the formulation assay of the developed method.

3. Method development

The development of HPLC methods for the determination of drugs has received great attention in analytical research because of their importance in the quality control. HPLC is the unique, versatile, universal, basic instrument and well utilized by the researchers because of its ease in the operation, availability and in terms of cost. In the present work, an attempt was made to develop a simple and rapid HPLC method for the routine analysis of Dabigatran in tablet formulations. For this purpose, the analytical column, solvent selection, mobile phase composition, flow rate, and detector wavelength were studied. The chromatographic columns changed in the analysis in order to find the appropriate column, Zodiac column, Chromosil column were used; mobile phase composition and flow rate was also carried to find the most suitable condition. The chromatographic separation was achieved using an RP Chromsil C18 column for Dabigatran with symmetrical sharp peak shape. Our experiments and data reported in the literature showed that both the methanol and acetonitrile could be used an organic modifier in the mobile phase. The use of acetonitrile as a mobile phase organic modifier resulted in better sensitivity compared to methanol. Mobile phase is varied with different proportions of methanol and acetonitrile to check for the best combination of mobile phase. The mobile phase composition of Methanol : Acetonotrile: Water in the ratio of 80:15:5 (v/v) was stated best for the analysis and estimation of Dabigatran, as the peak shape and retention time was suitable for the routine analysis of the drug.

The method has many advantages, e.g., simplicity, shorter run time, low injection volume, smaller particle size, and less flow rate, inexpensive mobile phases. Under these conditions, the retention time of Dabigatran was about 5.73min, with a good peak shape (peak tailing factor < 2), and the run time was 10min. Typical chromatogram of Dabigatran standard was shown in figure 2.

Table 1: Developed chromatographic conditions for the estimation of Dabigatran

Api Concentration	40µg/ml
Mobile Phase	Methanol:Acetonitrile: Water 80:15:5(v/v/v)
Wavelength	280nm
Column	Chromocil C18 column
P^H	4.7
Concentration	60µg/ml
Retention Time	5.73min
Flow rate	1.0ml/min
Run Time	10min
Area	390107.5
Th. Plates	5516
Tailing Factor	0.17
Pump Pressure	8.2±5MPa

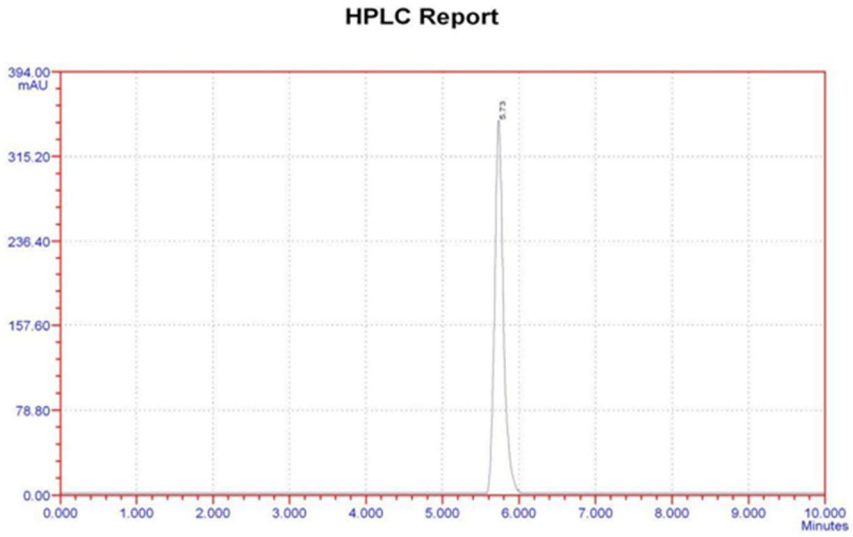


Figure 2: Standard chromatogram of Dabigatran

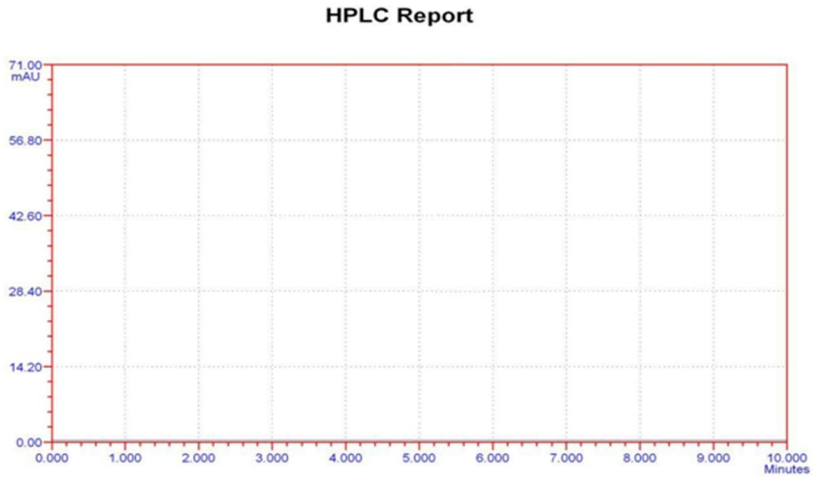


Figure 3: Blank chromatogram of Dabigatran

4. Method validation

It is axiomatic that experimental results obtained must give a good indication of true results. The calibration carried out in setting up the method go part of the way to ensuring this. Analysis of validated standards to check the performance of a method is almost always essential. In order to establish the performance characteristics of the method i.e. Linearity, precision, accuracy, ruggedness, robustness, LOD, LOQ were checked so as to meet the requirements of the intended analytical application. The primary objective of validation is to form a basis of process control which are designed to assure that the drug products have the identity, quality, strength and purity which they represent to possess.

According to ICH, typical analytical performance characteristics that should be considered for validation were; Linearity, precision, accuracy, ruggedness, robustness, LOD, LOQ and system suitability [6, 7].

4.1 System suitability

The system suitability test was applied to this chromatogram to check the various parameters such as, tailing factor, resolution, and repeatability against the specifications set for the method. The tailing factor must be below 2. Theoretical plates must be above2000.

4.2 Linearity

Solutions of different concentration in the range of (40 to 100 µg/ml) were prepared from stock solutions to plot a calibration curve to check the linearity of the method. Calibration curve was constructed by plotting Peak area Vs concentration of solution and chromatogram was shown in figure 4. Linearity was demonstrated at concentrations from 40 to 100µg/ml of the drug. The regression equation in the form of y=mx+c gives the slope, intercept and the correlation coefficient (r^2) depicts the degree of co-relation between the points of the beer's plot. The data meet the acceptance criteria for R^2 ≥ 0.999 and a y-intercept less than ± 2.0%, indicating a high degree of linearity.

4.3 Precision

Precision was considered at three different levels, i.e. repeatability, reproducibility and inter-mediate precision, in accordance with ICH recommendations. Repeatability of sample injection was determined as intra-day variation o the same day whereas inter-mediate precision was determined by measuring inter-day variation in consecutive days of a week for triplicate determination of Dabigatran at concentrations of 60µg/ml.

The precision of the method was validated by determination of inter day and intraday coefficients of variation (%RSD) and percentage deviation.

4.4 Accuracy

The difference between theoretical added amount and practically achieved amount is called accuracy of analytical method. Accuracy was determined at three different level 50%, 100% and 150% of the target concentration in triplicate. Result of accuracy data presented in Table
The recovery of the method, determined by spiking a previously analyzed test solution with additional drug standard solution.

4.5 Ruggedness

Ruggedness test was determined between two different analysts, instruments and Columns. The value of percentage RSD was below 2.0%, showed ruggedness of developed analytical method. The results were presented in Table

4.6 Robustness

The robustness of the method was determined by varying the method conditions slightly in the range of ±2 to the initial value of the condition specified such as changing the chromatographic condition of wavelength as 282 or 278nm, as the initial value of the wavelength suitable for the analysis is 280nm.

The robustness of the method is also depicted when small deliberate changes in the conditions were also facilitated like mobile phase composition and pH of the system.

4.7 Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were calculated using the following equations as per International Conference on Harmonization (ICH) guidelines. LOD was calculated using the formulae, LOD=S/N where Average Baseline Noise obtained from Blank was named as (S), Signal Obtained from LOD solution (0.25% of target assay concentration) was named (N).

The Quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated using the formulae, LOQ= S/N, where S was Average Baseline Noise obtained from Blank, N was Signal Obtained from LOD solution (0.75% of target assay concentration).

5. Stress degradation studies

In order to check the stability and degradation of the drug under different conditions, stress degradation studies have been conducted on the drug.

5.1 Degradation under acid catalyzed hydrolytic condition

1 ml of working standard solution of Dabigatran of 100mg/ml was mixed with 1 ml of 5 N HCl. The solution was diluted to 10 ml with methanol and kept for 4 hours. Appropriate volume of resultant solution 10µl was injected after 48hours and degradedness in the chromatogram was determined.

5.2 Degradation under alkali catalyzed hydrolytic condition

1 ml of working standard solution of Dabigatran of 100 mg/ml was mixed with 1ml of 1N NaOH. The solutions were diluted to 10 ml with methanol and kept for 4 hours. Appropriate volume of resultant solution was injected in the column after 48hours and degradedness in the chromatogram was determined.

5.3 Degradation under oxidative conditions

In order prepare oxidized sample at zero hours and after 48 hours. Take 300 mg sample in 20 ml of 3% Hydrogen Peroxide. After 48 hours to take 5 ml of oxidized sample solution in 25 mL volumetric flask and make up with diluent. The above solutions inject once after system suitability solution and evaluate the degradants in chromatogram and compare with without oxidized (Initial) values.

5.4 Photolytic degradation

To demonstrate the degradation of the sample, keep in open petri dish at Lab light and UV light. Check the sample at 48 hours exposed sample at Lab light and UV light. Prepare sample solutions of the above, inject once in the chromatographic conditions. Evaluate the degradants in chromatogram and compare for degradedness.

5.5 Thermal degradation

To find the degradation of the drug sample keep in oven at 40°C and 80°C up to 48 hours. After exposure of the samples to high temperatures, then it inject once after system suitability has been performed. Evaluate the chromatogram for the degradants.

5.6 Hydrolytic degradation

To evaluate the degradation in aqeous condition take 300 mg sample in 20 ml of Aqueous solution. After 48 hours take 5 ml of sample solution in 25 mL volumetric flask and make up with diluent. The above solutions inject once after system suitability solution and evaluate the degradants in chromatogram and compare with without aqueous values.

6. Results and discussions

6.1 Method development

In the present study, a simple, selective, rapid and specific have been devised to estimate Dabigatran in pharmaceutical tablet dosage forms. Different trails have been performed in order to develop suitable methods for the analysis. Column selection was done basing upon the polarity of the drug and interaction between the column and the analyte. It has been found after numerous trails mobile phase composition was finalized as Methanol: Acetonitrile: Water 80:15:5(%v/v/v). The pH of the system was maintained as 4.7 and optimum flow rate for elution of the drug was found to be 1.0ml/min. The UV detection wavelength was set as 280nm, as from the UV absorption spectrum of the drug in UV spectrophotometer.

6.2 Method validation

The developed method was validated according to ICH guidelines. It has been stated by the results and respective values within the acceptable range the proposed method is linear, precise, accurate, robust, rugged, sensitive and it was also a stability indicating method. From the stress degradation studies it has been proved that the drug was stable up to 24hours. Results of validation parameters were illustrated below.

6.2.1 Linearity

The linearity of an analytical procedure is the ability to obtain test results that are directly proportional to the concentration of an analyte in the sample. The calibration curve showed good linearity in the range of 40-100 µg/ml, for Dabigatran (API) with correlation coefficient (r^2) of 0.999. A typical calibration curve has the regression equation of y =6450x-3297.38. Results are given in Table 1.

Table 2: Linearity results of Dabigatran

S.NO	Concentration in µg/ml	Peak Area
1	40	248683
2	50	322218
3	60	390107
4	70	441663
5	80	508477
6	90	567919
7	100	655135
	Slope **Intercept** **Cc**	**6450.165** **-3297.38** **0.999**

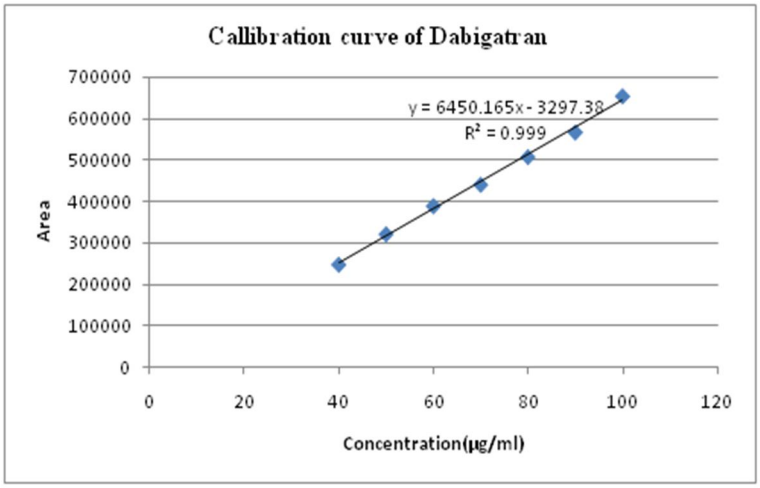


Figure 4: Calibration curve of Dabigatran

6.2.2 Precision

The Precision of the method was studied in terms of intraday and interday precision of sample injections (5µg/ml). Intraday precision was investigated by injecting three replicate samples of each of the sample on the same day. The % RSD was found to be 0.39. Interday precision was assessed by analysis of the same solutions on consecutive days. The % RSD obtained was found to be 0.56. Low % RSD values indicate that the method is precise. The results are given in table 3 and 4.

Table 3: Intraday Precision

S.NO	Concentration in µg/ml	Peak Area
1	60	392761
2	60	394435
3	60	391397
4	60	391227
5	60	391271

6	60	394518
	% RSD	**0.39**

Table 4: Interday Precision

S.NO	Concentration in µg/ml	Peak Area
1	60	382054
2	60	385033
3	60	382879
4	60	384989
5	60	388186
6	60	385740
	% RSD	**0.56**

6.2.3 Recovery

The accuracy of method was determined by recovery, by spiking of standard drug solution to pre analyzed sample at three different levels i.e., at 50, 100, and 150%. The resultant solutions were then re-analyzed by the developed method. At each concentration, sample was injected thrice to check repeatability and from the data it was analyzed that method was accurate as % recovery values found to be in the range of 98.62 % to 99.81% at three different concentrations 60, 80 and 100µg/ml.

Table 5: Recovery results of Dabigatran

% of Recovery	Dabigatran					
	Target Conc., (µg/ml)	Spiked conc., (µg/ml)	Final Conc., (µg/ml)	Conc., Obtained	% of Assay	%RSD
50%	40	20	60	59.72	99.54	0.43
	40	20	60	59.21	98.69	
	40	20	60	59.38	98.97	
100%	40	40	80	79.55	99.43	0.45
	40	40	80	78.93	98.66	
	40	40	80	78.90	98.62	
150%	40	60	100	99.32	99.32	0.25
	40	60	100	99.47	99.47	
	40	60	100	99.81	99.81	

6.2.4 Ruggedness

The method was validated by performing the analysis by different chemists, in order to check the repeatability and to minimize the human errors. The RSD value 0.76 illustrates that method was suitable to analyze different drugs as values were in order of repeatability depicting the precision of the method.

Table 6: Ruggedness results of Dabigatran

S.NO	Concentration in µg/ml	Peak Area
1	60	393970
2	60	390330
3	60	399126
4	60	395390
5	60	392093
6	60	393195
	% RSD	**0.76**

6.2.5 Robustness

Small deliberate changes in chromatographic conditions such as change in mobile phase ratio (± 10 ml), change in pH (± 1) and detection wavelength of (± 2 nm) were studied to determine the robustness of the method. The results were in favor of (% RSD < 2%) the developed RP-HPLC method for the analysis of Dabigatran. The results are given in table 7.

Table 7: Robustness results of Dabigatran

Condition	Mean area	% difference
Standard	**390107**	
Mp Changes 75:20:5	386108	-1.02
Mp Changes85:10:5	384305	-1.48
WL Changes278nm	387701	-0.61
WL Changes282nm	392725	0.67
PH changes 4.5	387567	-0.65
PH changes4.9	386634	-0.89

6.2.6 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ of Dabigatran were calculated by mathematical equation and were found to be 0.25 µg/ml, 0.8 µg/ml respectively.

6.2.7 Stability indicating studies

The stress degradation studies suggest that drug stability was 20%-80% establishing stability indicating nature of the method. RP-HPLC study of samples obtained on stress testing of Dabigatran under different conditions using mixture of methanol, acetonitrile and water in the ratio 80:15:5 (%v/v/v) as a mobile solvent system suggested the following degradation behavior. It has been concluded that the drug was stable upto 24hours and it degrades into different compounds in the due course. The chromatograms obtained by injecting drug solution undergone with stress conditions were shown in the figures

Table 8: Results of degradation studies

Condition	Report
Base	Main peak split into 4 peaks
Light	Main peak split into 2 peaks
Peroxide	Main peak split into 4 peaks
UV light	Main peak split into2 peaks
Thermal	Main peak split into 4 peaks
Acidic	Main peak split into 8 peaks
Aqueous	Main peak split into 5 peaks

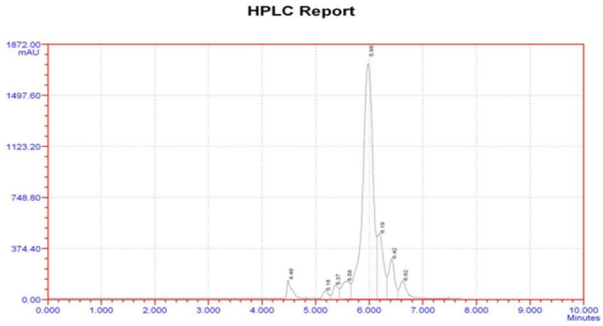


Figure 5: Acid hydrolysis

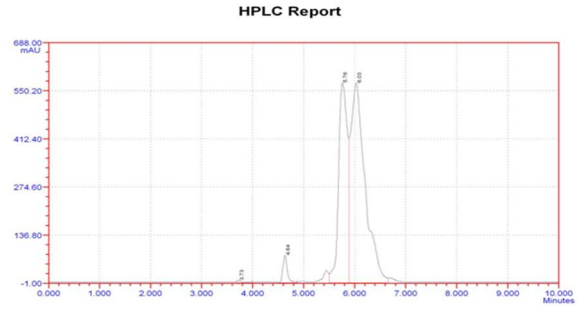
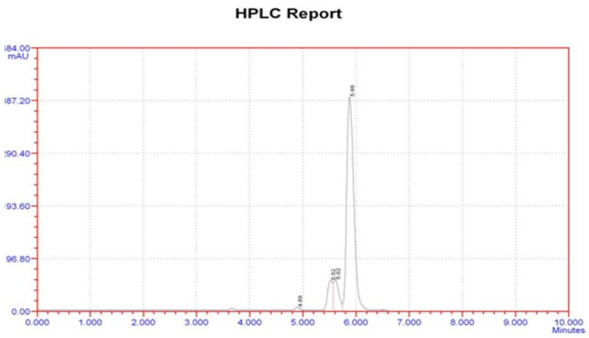


Figure 6: Oxidative degradation



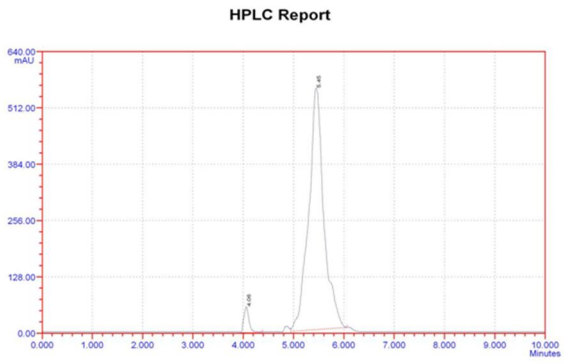
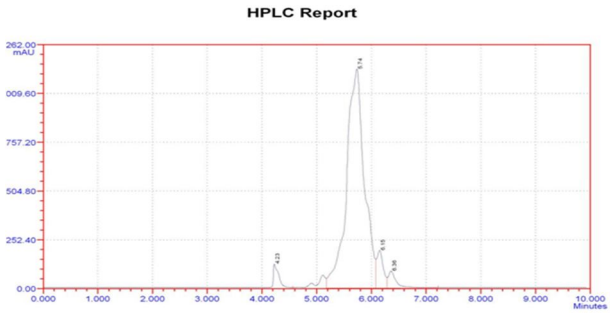


Figure 7: Base hydrolysis

Figure 8: Photolytic degradation



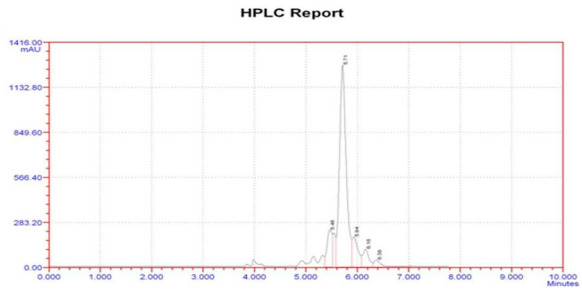


Figure 9: Thermal degradation

Figure 10: Aqueous degradation

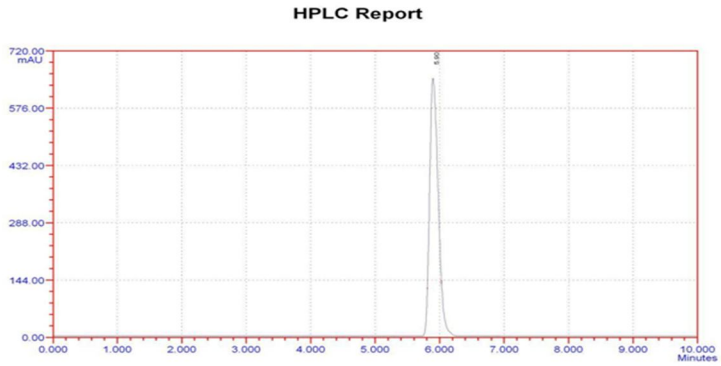


Figure 11: Zero hour chromatogram

7. CONCLUSION

HPLC method was developed and validated as per ICH guidelines. UV detection of 280nm allowed an accurate quantization of drug in pure and tablet dosage form. The drug was analyzed by HPLC method using Chromocil C-18 Column (4.6 x 250 mm, 5µm), with isocratic conditions and simple mobile phase containing methanol:acetonitrile:water (80:15:5) v/v at flow rate of 1.0ml/min . The procedure has been evaluated for the linearity, accuracy, precision and robustness in order to ascertain the suitability of the analytical method. The method was also applied to marketed samples. It has been proved that the method is selective and linear between concentration range 40-100µg/ml of for. LOD and LOQ were found to be 0.25µg/ml and 0.8µg/ml. It can be concluded from degradation studies the method is specific as the retention time of the drug were unaffected by degradation products. The present study reveals that the method developed can be used for routine analysis of Dabigatran in pure and pharmaceutical formulations.

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