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Development and Validation of the Reversed Phase UPLC Method for the Quantitative Estimation of Dabigatran Etexilate in Dabigatran Etexilate Capsules

Sanjay S Shetgar[1*], K Basavaiah[2] and B. M Rao[3]
[1]Dept. of Inorganic and Analytical Chemistry, Andhra University, Visakhapatnam - 530003, Andhra Pradesh, India.
[2]Professor, Dept. of Inorganic and Analytical Chemistry, Andhra University, Visakhapatnam - 530003, Andhra Pradesh, India.
[3]Vice President & Head ASAT & Corporate Quality Control, Dr. Reddy's Laboratories, Hyderabad - 500090, Telangana, India.

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***Corresponding Author Email:** sanjayshetgar@yahoo.com

Abstract
Current work discloses development and validation of a simple, accurate, sensitive, and quick reverse phase ultra-performance liquid chromatograph (RP-UPLC) method for the quantitative estimation of Dabigatran Etexilate in Dabigatran Etexilate capsules. This development was achieved using a Waters Acquity UPLC with Hibar C18 column of dimensions 100 mm x 2.1 mm, 1.8µm column at 0.3 mL/min flow rate and Acquity TUV detector at 218 nm. This analytical UPLC method is validated based on the guidelines of the International Conference on Harmonization (ICH – Q2(R2), November 2005). Linearity was demonstrated in the range 25% to 150% levels with Coefficient of Determination (R^2) value of 0.9999. Precision and Accuracy performed using the ICH guidance approach with recovery at 100.07%. The RP-UPLC method is sensitive with levels of Limit of detection (LOD) and Limit of Quantitation (LOQ) at 0.82µg/mL and 2.50µg/mL respectively. Degradation studies in conditions of Oxidation, Acid, Alkali, Thermal, Photostability and Water demonstrate no interference from the degradants. This simple, accurate, sensitive and quick RP-UPLC method for Dabigatran serves as an efficient tool in routine quality control and stability testing of Dabigatran and its formulations.

Keywords:
Accuracy, Dabigatran, Limit of Detection, Limit of Quantitation, Reversed phase Ultra Performance Liquid Chromatography

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1.0 INTRODUCTION:
Dabigatran Etexilate Mesylate is thrombin inhibitor and demonstrates anticoagulant activity. The drug substance Dabigatran Etexilate Mesylate has limited oral availability and is absorbed as Dabigatran Etexilate ester. On administration Dabigatran Etexilate is hydrolyzed by the enzyme esterase and is converted to Dabigatran. Dabigatran inhibits the activity of thrombin, a serine protease that converts fibrinogen into fibrin. Due to this the coagulation cascade is interrupted and in turn prevents the formation of blood clots. Dabigatran Etexilate Mesylate (Figure1), chemically known as β-Alanine, N-[[2-[[[4-[[[(hexyloxy) carbonyl] amino]

iminomethyl] phenyl] amino] methyl]-1-methyl-1H-benzimidazol-5-yl] carbonyl]-N-2-pyridinyl-ethyl ester, methane sulfonate. Literature review for analytical estimations reveals that different analytical methods have been used for determination of Dabigatran. These methods include LC-MS, HPLC and UPLC methods [1-9]. The objective of this study is to develop a simple, accurate, sensitive and quick method for the quantitative estimation of Dabigatran Etexilate in Dabigatran Etexilate capsules and validate it in accordance to ICH guidance so that it can be applied for routine as well as stability indication analysis in the quality control laboratories.

2.0 MATERIALS AND METHODS:

2.1 Materials:

Dabigatran Etexilate Mesylate pure drug (API) and Dabigatran Etexilate Capsules (DABIREX 110 mg) was obtained as gift samples from Dr. Reddy's Laboratories. Solvents like acetonitrile, methanol were HPLC grade solvents and were procured from manufacturer Rankem. Other reagent of analytical reagent grade like potassium dihydrogen ortho phosphate, ortho-phosphoric acid and glacial acetic acid were procured from Rankem. Purified water for chromatographic work was obtained with Milli Q Water filtration system.

2.2 Equipment:

Waters-Acquity UPLC system with Auto Injector and tunable UV Detector and Waters Empower 2 chromatographic data system along with sonicator (Ultrasonic), pH meter (Thermo scientific), Micro balance (Sartorius) and vacuum filter pump.

2.3 Method:

2.3.1 Chromatographic conditions:

The chromatographic system used was Waters Acquity UPLC with Hibar C18 column of dimensions 100mm X 2.1mm, 1.8µm column. Mobile phase composition comprised of 0.1% Orthophosphoric acid Buffer (pH 2.5) and Acetonitrile in the ratio 70:30. Flow rate was set at 0.3mL/minute with 0.3µL injection volume and column oven temperature of 30°C. Diluent used was a mixture of 0.1% Orthophosphoric Acid buffer and Acetonitrile in the ratio 50:50 for preparations of standard and sample preparations. Detection was achieved with Waters Acquity TUV detector at 218nm. Instrument operation, chromatographic data acquiring and processing was done using Waters Acquity UPLC with Empower 2 software.

2.3.2 Mobile Phase Preparation:

2.3.2.1 Preparation of 0.1% Orthophosphoric acid Buffer (pH-2.5): 1mL of ortho phosphoric acid was diluted to 1000mL with purified water from Milli-Q Water filtration system.

2.3.2.2 Diluent: Diluent was selected based on the solubility of the drug Dabigatran Etexilate. 0.1% Orthophosphoric acid buffer (pH 2.5) and Acetonitrile was taken in the ratio of 50:50.

2.3.2.3 Mobile Phase: 0.1% Orthophosphoric acid buffer (pH 2.5) and Acetonitrile in the ratio 70: 30

2.3.3 Standard Preparation:

Preparation of Standard stock solutions**:** Stock solution having concentration of 1100 µg/mL of Dabigatran was prepared by dissolving in diluent with aid of sonication. For injecting into the chromatograph diluted standard working solutions was prepared by diluting 1mL of Dabigatran from each stock solution to 10 mL with diluent to get 110 µg/mL of Dabigatran.

2.3.4 Sample Preparation:

Preparation of Sample stock solutions: 5 capsules were weighed and contents powdered. Weight equivalent to 1 capsule was transferred into a 100 mL volumetric flask, 50mL of diluent was added and sonicated for 25 minutes, further the volume was made up with diluent and filtered by 0.45 microns PVDF filters (1100 µg/mL of Dabigatran). For injecting into the chromatograph diluted sample working solution was prepared by diluting 1mL of filtered sample stock solution to 10mL with diluent to get 110 µg/mL of Dabigatran.

3.0 RESULTS AND DISCUSSION:

3.1 Optimization of sample preparation

The sample preparation needs to ensure complete extraction from the formulation mix. Based on solubility of the Dabigatran, acetonitrile and phosphate buffer combination were assessed and optimized for efficient extraction. Filtration of sample solution was done using 0.45 micron PVDF filter. There was no retention of the drug on the filter.

3.2 Optimization of chromatographic conditions

The sensitivity of the UPLC method depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for all impurities and analyte peak to be detected. The wavelength for measurement was selected as 218 nm based on the UV maxima exhibited in the absorption spectrum (Figure 2). Various UPLC columns like BEH, HSS and Hibar with 50 mm and 100 mm lengths and mobile phase options of 0.1% orthophosphoric acid and acetonitrile mixture and 0.1% triethylamine and acetonitrile mixture options were evaluated. Usage of 0.1% triethylamine and acetonitrile mixture led to poor peak shape and plate count. This was optimized with use of 0.1%

orthophosphoric acid and acetonitrile mixture. Acetonitrile was used as it provided better analyte response. Final optimized conditions were achieved using Waters Acquity Hibar C18 column of dimensions 100mm X 2.1mm, 1.8µm with flow rate of 0.3mL/minute and column oven temperature of 30°C.

3.3 Method Validation

For the newly developed RP- UPLC method, validation was performed according to the ICH guideline for the analytical parameters such as system suitability, specificity, linearity, precision, intermediate precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and robustness in order to demonstrate the adequacy of the method.

3.3.1 System suitability parameters:

The system suitability parameters were determined by preparing standard solutions of Dabigatran standard (110µg/mL) and the solutions were injected as six replicates and the parameters like % RSD, peak tailing and USP plate count were determined. The % RSD was below 2%. Retention time variation was observed in range 0.968 – 1.008 minutes. Plate count was ranging from 2305 to 2371. The Chromatographic parameters such as % RSD of six replicate dilute standard injections, Number of theoretical plates and tailing factor for Dabigatran are tabulated under Table-1.

3.3.2 Specificity:

Specificity for the RP-UPLC method was demonstrated by no interference from the blank, placebo and potential degradation impurities from the degradation study conducted in line with the ICH guidance. Maximum degradation to level of 8.8% was achieved using the base conditions. Chromatogram demonstrating separation of the Dabigatran from the potential impurity peaks is shown in Figure 3. The degradation of the drug Dabigatran under the ICH specified degradation conditions is tabulated in Table 2. Chromatogram for specificity was reviewed for any interference from the blank, placebo and potential degradation impurities. There were no interferences of any of the potential degradants at the retention time of the Dabigatran peak. Chromatograms are shown in Figure 4 to Figure 6

3.3.3 Precision:

The precision of the RP-UPLC assay method as part of repeatability was checked by six replicate injections of Dabigatran sample solutions of 110 µg/mL concentration. The % amount of Dabigatran was calculated and RSD was found to be 1.2%. Details of Precision repeatability are presented in Table 3.

Intermediate precision was established by injecting five sample solutions of concentration 110µg/mL of Dabigatran. These were injected into the chromatograph in the optimized chromatographic conditions after 24 hours of preparation. The % Amount of Dabigatran found was calculated and %RSD was found to be 1.2%. Details of Intermediate precision are presented in Table 3.

3.3.4 Linearity:

To demonstrate the linearity of Dabigatran RP-UPLC method, Dabigatran standard solution of concentration 1100µg/mL was prepared. Using this stock, 6 standard solutions with concentrations at 25%, 50%, 75%, 100%, 125% and 150% corresponding to 27.5µg\mL, 55 µg\mL, 82.5µg\mL, 110µg\mL, 137.5 µg\mL and 165µg\mL of Dabigatran were injected in the RP-UPLC system with the optimized chromatographic condition. Concentration versus peak area was plotted and regression analysis was performed. The correlation co-efficient was found to be 0.9999 with 95 % confidence interval. Details of concentration and peak area are shown in Table 4. Linearity plot is presented in Figure 7.

3.4.5 Accuracy:

To demonstrate the accuracy of the Dabigatran RP-UPLC assay method Dabigatran sample solution of concentration 1100 µg/mL was prepared by taking one capsule equivalent of the powdered Dabigatran capsule contents followed by sonication with the diluent and filtration. Known fixed quantities of Standard stock solution were spiked on to Sample solution at levels of 50%, 100%, and 150%. Each of the sample solution spiked with known concentration of the standard (50%, 100%, 150% of Dabigatran) was injected in triplicate in the optimized chromatographic system and % recovery was observed to be 100.07%. Accuracy data is tabulated as Table 5.

3.4.6 Limit of Quantitation (LOQ) AND Limit of Detection (LOD):

The LOQ and LOD were established by injecting dilute solutions of dabigatran and measured the signal to noise ratio. The LOD and LOQ values were observed to be at 0.82 µg/mL and 2.5µg/mL respectively. Chromatograms are shown in Figure 8 and Figure 9 respectively.

3.4.7 Robustness:

Robustness of the RP-UPLC Assay method was demonstrated by small deliberate changes in the method which could potentially occur in a laboratory environment by going on either side of the chromatographic parameters like Flow (±0.3ml/min); Mobile phase (±2%); Temperature (±5°C). Under each of these conditions sample preparations were injected in duplicate. The system suitability parameters and amount of the Dabigatran content was calculated and found comparable. The variation

in RSD of the Dabigatran content in the listed conditions was observed to be in the range 0.4% to 1.8%. Details are tabulated in Table 6.

3.4.8 Assay of the marketed formulation:

Assay of the Dabigatran Etexilate capsule sample batch was performed in line with the new developed RP-UPLC assay method. Standard and sample solutions were prepared at a concentration of 110µg/mL of Dabigatran and chromatographed using the RP-UPLC Assay method. The results were observed to be 99.64% with RSD at 1.16%. The area count, % assay and RSD are tabulated in Table 7. Chromatogram for standard and sample are shown in Figure 8 and Figure 9.

Figure 1: Chemical Structure for Dabigatran Etexilate Mesylate

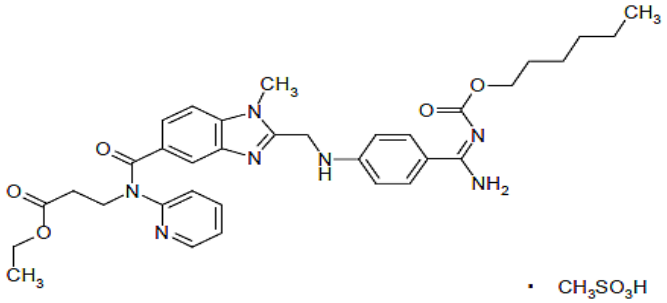

Figure 2: UV Absorption Spectrum of Dabigatran Etexilate Mesylate

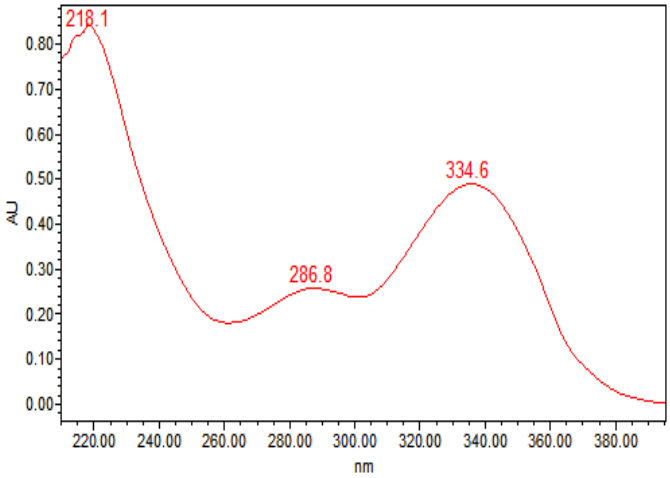

Figure 3: Forced Degradation - Base Condition

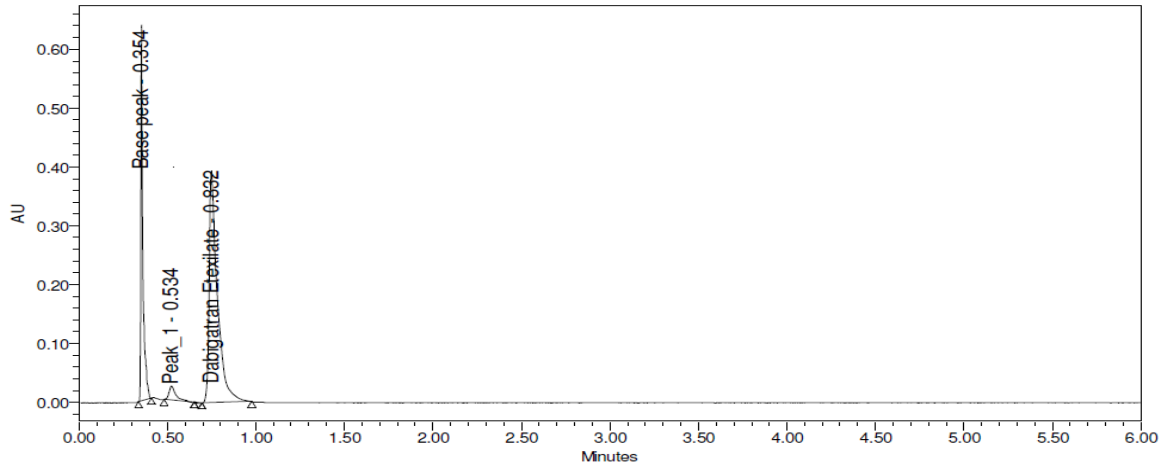

Figure 4: Blank Chromatogram

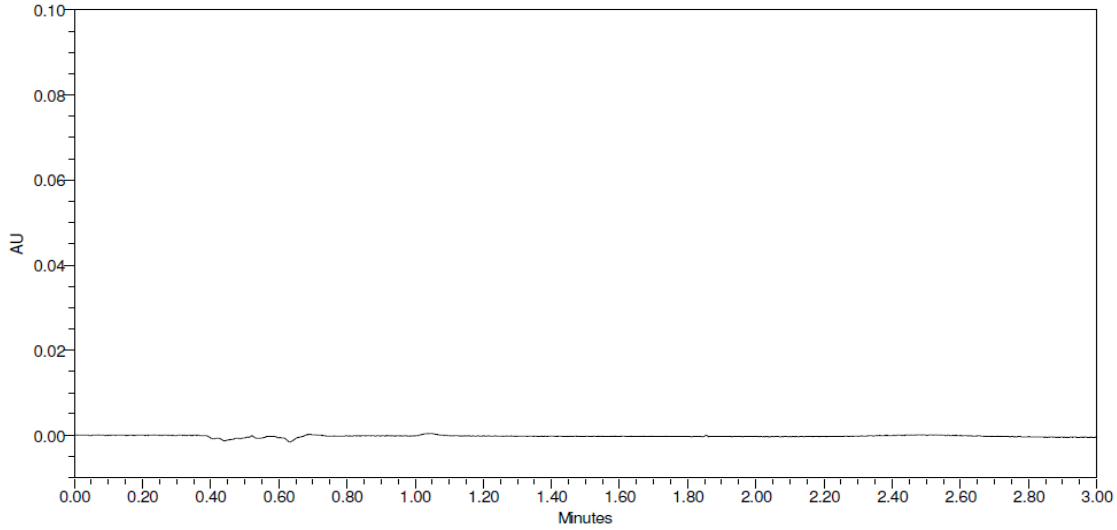

Figure 5: Placebo Chromatogram

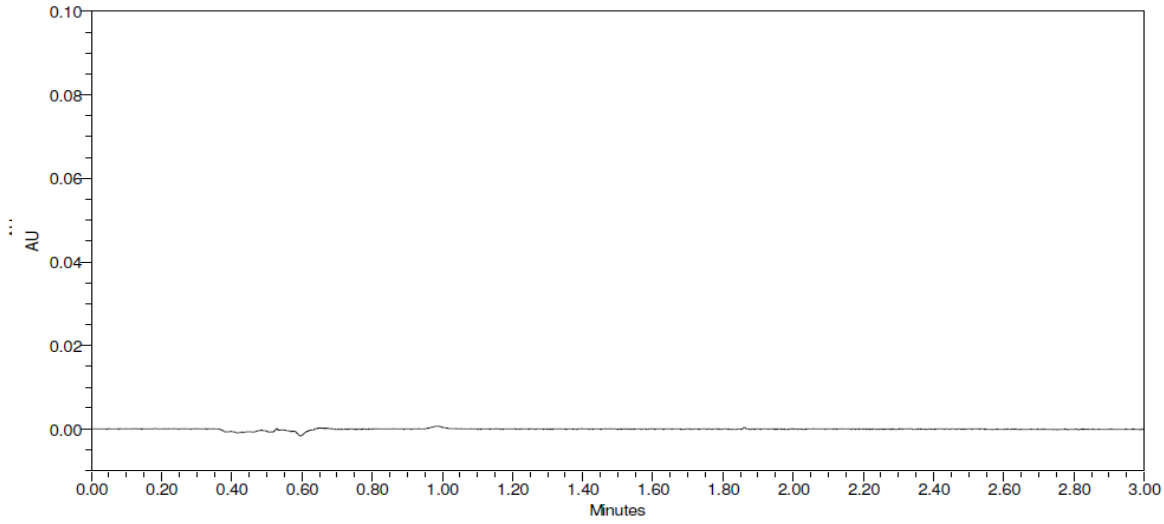

Figure 6: Typical Chromatogram for Dabigatran

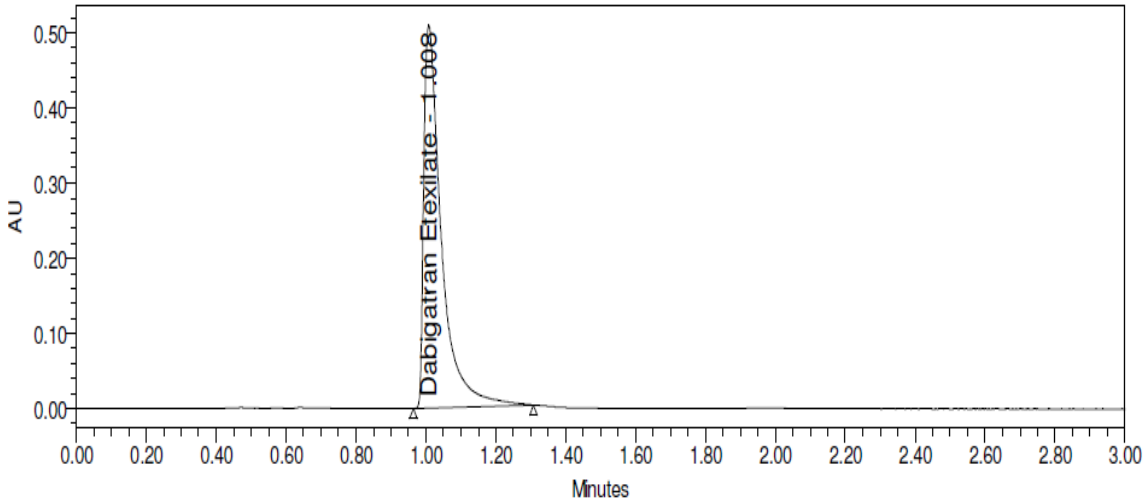

Figure 7: Linearity Plot for Dabigatran Assay Method

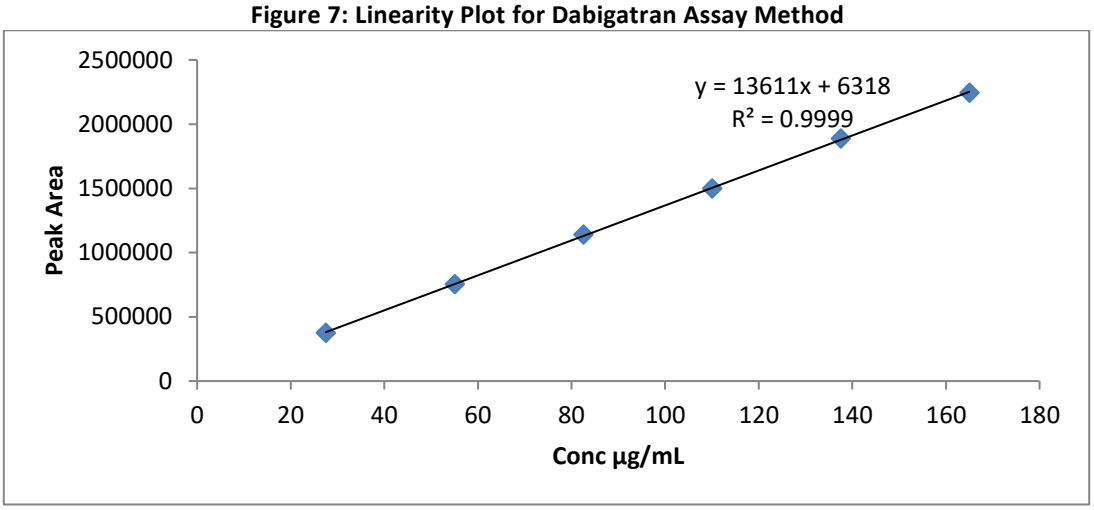

Figure 8: Chromatogram for Limit of Detection (LOD):

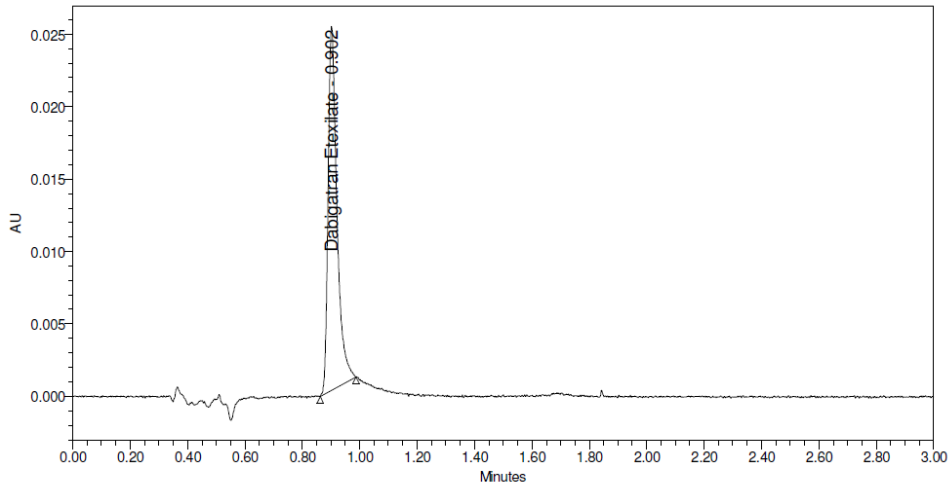

Figure 9: Chromatogram for Limit of Quantification (LOQ):

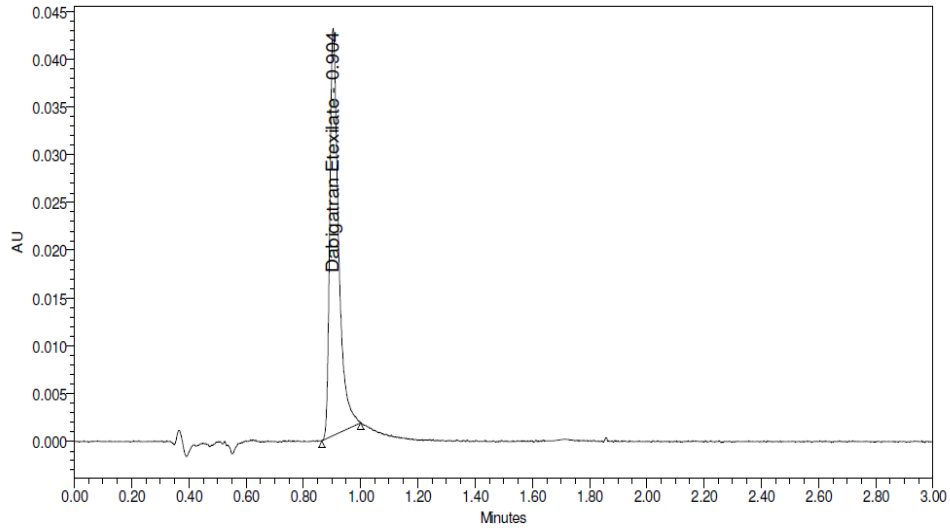

Figure 10: Dabigatran Etexilate Standard Chromatogram

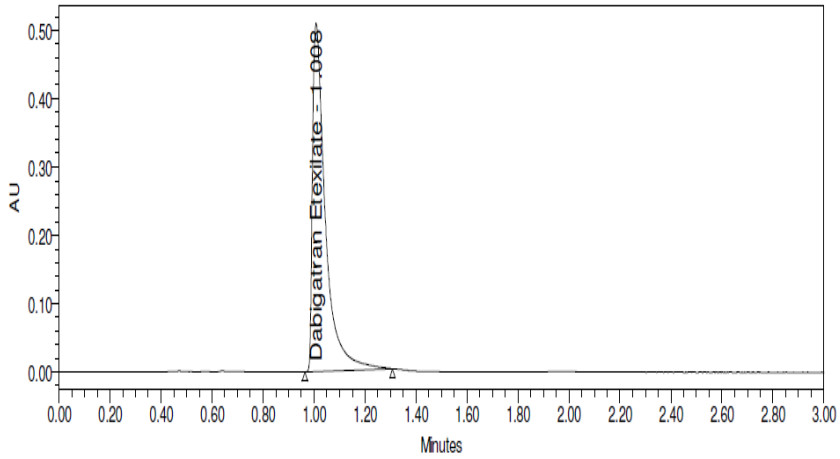

Figure 11: Dabigatran Etexilate Sample Chromatogram

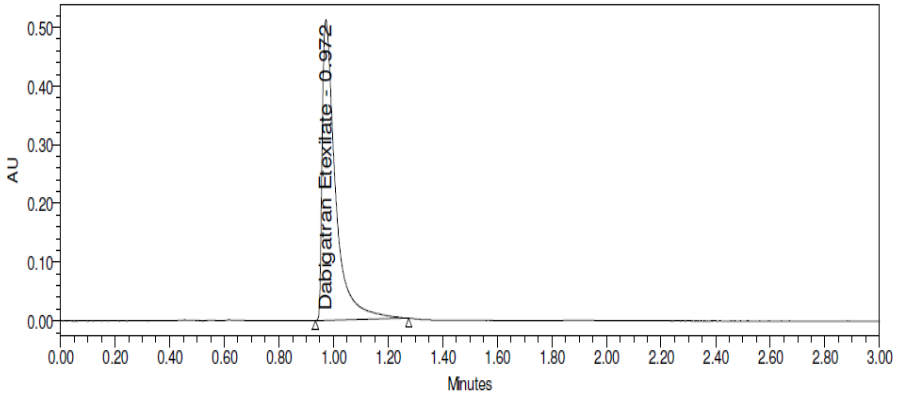

Table 1: Degradation Data of Dabigatran Etexilate Capsules

S.No	Degradation Condition	% Drug Undegraded	% Drug Degraded
1	Acid (2 N HCl, Reflux 30 min, 60°C)	94.25	5.75
2	Alkali (2 N NaOH, Reflux 30 min, 60°C)	91.23	8.77
3	Oxidation (20% H_2O_2, 30 min, 60°C)	96.70	3.30
4	Thermal (6 hrs,105°C)	98.02	1.98
5	Photostability (UV light 200 Watt hour/m^2, 7 days)	99.03	0.97
6	Water (Water, Reflux 6hrs, 60^0C)	99.53	0.47

Table 2 – System Suitability Parameters – Dabigatran Assay method

S no	Dabigatran		
Inj	**Retention Time (min)**	**USP Plate Count**	**Tailing**
1	0.968	2357	1.73
2	0.981	2368	1.79
3	0.986	2371	1.77
4	1.007	2338	1.71
5	1.008	2305	1.72
6	1.008	2331	1.71
Avg	**0.993**	**2345**	**1.74**
SD	**0.02**	**25.27**	**0.03**
%RSD	**1.7**	**1.1**	**1.9**

Table 3: Precision - Repeatability and Intermediate Precision

Injection	Repeatability Dabigatran Peak Area	Intermediate Precision Dabigatran Peak Area
1	1489954	1467169
2	1490664	1456803
3	1521975	1501146
4	1521048	1454622
5	1504167	1457197
6	1531662	1473995
AVG.	1509912	1468489
STD DEV	17575.0	17634.6
% RSD	1.2	1.2

Table 4: Method Linearity Dabigatran Concentration and Area response

Linearity Level (%)	Dabigatran Concentration (µg\ml)	Peak Area
0	0	0
25	27.5	374580
50	55	755088
75	82.5	1139101
100	110	1498242
125	137.5	1888529
150	165	2242846

Table 5: Accuracy - % Recovery of Dabigatran

% Level	Amount of Dabigatran Standard Spiked (µg/mL)	Amount of Dabigatran recovered (µg/mL)	% Dabigatran Recovery	Mean Dabigatran % Recovery
	55	55.90552	101.65	
50%	55	55.14929	100.27	
	55	54.43325	98.97	
	110	110.4111	100.37	
100%	110	108.8442	98.95	100.07%
	110	109.7819	99.80	
	165	167.6703	101.62	
150%	165	166.5451	100.94	
	165	161.8461	98.09	

Table 6 - Robustness

Parameter Varied	% RSD for Dabigatran
Flow Reduced to 0.27ml/min	0.9
Flow Increased to 0.33ml/min	0.4
Mobile phase Ratio Reduced (75B:25A)	1.2
Mobile phase ratio Increased (65B:35A)	1.8
Temperature Reduced to 25^0C	1.4
Temperature Increased to 35^0C	1.2

Table 7: Assay of Dabigatran Etexilate Capsules

Sample No	Dabigatran Standard Area	Dabigatran Sample Area	% Dabigatran (Assay)
1	1495489	1489954	98.3
2	1495651	1490664	98.4
3.	1518504	1521975	100.4
4.	1493654	1521048	100.4
5.	1534876	1504167	99.3
6.	1526621	1531662	101.1
AVG	1510799	1509912	99.64
STDEV	18150.6	17575.0	1.16
%RSD	1.2	1.2	1.16

4.0 CONCLUSIONS:

In conclusion, a simple, accurate, sensitive and quick method was developed and validated for the estimation of Dabigatran Etexilate in Dabigatran Etexilate Capsules by RP-UPLC technique. System suitability parameters were studied by injecting six replicates of the standard with RSD below 2.0%. Linearity study was carried out between 25% to 150% levels, Coefficient of determination (R^2) at 95 % confidence interval was observed to be 0.9999 indicating good correlation. Precision was found to be at an RSD of 1.2% for both repeatability as well as intermediate precision. LOD and LOQ were established at 0.82 µg/mL and 2.50 µg/mL respectively. % recovery of Dabigatran was found to be 100.07%. By using the validated RP-UPLC Assay method the marketed formulation was tested and observed to be at 99.64%. Degradation studies of Dabigatran Etexilate capsules conducted on lines on ICH demonstrated that the impurity peaks didn't interfere with the Dabigatran peak hence indicating that method is stability indicating. The developed method hence can be conveniently applied for the quantitative estimation of Dabigatran in the API as well as the finished drug product in routine and stability indicative evaluations. The use of UPLC methods would also go a long way to support the laboratory efficiency which is the need of the hour.

5.0 ACKNOWLEDGEMENTS:

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