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Der Pharmacia Sinica, 2015, 6(4):91-102

Der Pharmacia Sinica
ISSN: 0976-8688
CODEN (USA): PSHIBD

Quantification of anticoagulant drug substance of Dabigatran Etexilate by a validated Ultra performance liquid chromatographic method with detailed force degradation study

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ABSTRACT

The aims of this study to develop a method and validated it for Dabigatran etexilate which is used as an anticoagulant agent. Here we reported sensitive, precise, an accurate and time saving stability indicating methods performed on UPLC. The study was performed using HSS C18 (50 x 2.1 mm i.d.) column having 1.8 µm particle size, with buffer: acetonitrile (50:50 v/v) mobile phase. The assay method was carried out at 225 nm wavelength with PDA detector. The method was validated using parameters such as accuracy, precision, linearity, robustness, solution stability and degradation in different stress conditions.

Key words: Dabigatran etexilate, Stability-indicating, Force Degradation, UPLC.

INTRODUCTION

Dabigatran Trade name Pradaxa is an antithrombics drug from the class of direct thrombin inhibitors. Dabigatran etexilate chemically known as Ethyl 3-{[(2-{[(4-{N'-hexyloxycarbonyl carbamimidoyl}phenyl)amino]methyl}-1-methyl-1H-benzimidazol-5-yl)carbonyl] (pyridin-2- yl-amino)propanoate. [1, 2] (Fig. 1)

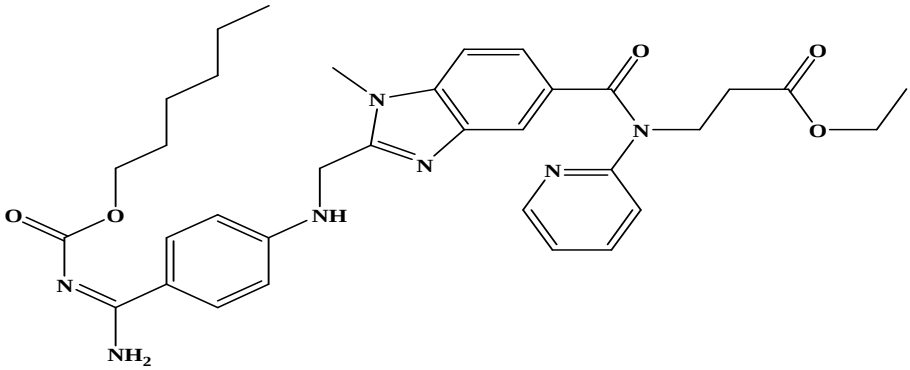

Fig. 1: Chemical structure of Dabigatran etexilate

Antithrombics drugs are being used for delaying blood clotting. These groups of drugs are considered to use for treatment of thrombotic disorders. Anticoagulants drugs are directly inhibiting enzyme Thrombin. Dabigatran etexilate is metabolite as Dabigatran in body. Inhibition of enzyme thrombin inhibits coagulation cascade and as a

results prevention of clots. [1,3,4,5] Dabigatran etexilate is used to prevent stroke and blood clot formation in patients who have suffered from Artrial Fibrillation. Dabigatran is a well known alternative anticoagulant drug for the patients of Atrial fibrillation to avoid the side effects of Warfarine. Dabigatran etexilate also useful in the treatment of Venus thromboembolism at the time of elective Hip and Knee surgery.[6-10]

Literature review of Dabigatran etexilate, it is reveals that various analytical methods were previously published for quantification of Dabigatran etexilate by different spectrophotometric and chromatographic methods.[11-18] However all the methods of quantification of dabigatran developed and validated by HPLC. Which are time consuming and lack of reproducibility. To avoid above cited draw back we developed accurate, precise and time saving chromatographic method by UPLC. This method also indicates actual storage of this drug. In literature of all available methods degradation was done at higher temperature. In fact drug is very much sensitive; we found that drug start degrading at room temperature with specific stress conditions. Thus there was no need to apply high temperature as a secondary force condition. We performed degradation study using various primary stress conditions like different concentrations of acid and base for certain period. The purpose of this work was developed and optimized a fully validated stability indicating and routinely available ultra performance liquid chromatographic method as per ICH Guidelines. [19, 20, 21]

MATERIALS AND METHODS

Chemical and Reagents

Dabigatran etexilate (Potency 99.70) was purchased from Amney Pharmaceuticals. HPLC-grade Acetonitrile and all other chemicals were purchased from Spectrochem Laboratories. HPLC-grade water was prepared by using a Milli-Q system, Millipore Corp. and it was used for all purposes.

Chromatographic condition:

The protocol for quantification of Dabigatran etexilate in drug substance has been performed on Waters Acquity UPLC system includes a binary solvent manager, a sample manager, PDA detector and software using Empower 2.0 version for data acquisition. HSS C18 (50 X 2.1 mm i.d., 1.8 µm particle size) column has been used for Chromatographic Separation. The mobile phase used for the Isocratic elution was Buffer: Acetonitrile (50:50 v/v) with a composition of Buffer: 0.1% Triethylamine in water, at pH 5.0 ± 0.05 in Glacial acetic acid and 0.35 ml/min flow rate, while the injection volume was 2.0 µl. The mobile phase was filtered through 0.22 µ filter paper. The absorption maxima of Dabigatran etexilate was obtained at 225 nm wavelength. The protocol has been performed by keeping Column temperature 30 °C. The total elution time was 3.0 min.

Preparation of Standard solution:

Standard Solution was prepared by dissolving 25 mg working standard of Dabigatran etexilate (potency 99.70) in 50 ml volumetric flask. The final standard solution with 25 µg/ml concentration has been prepared by 2.5 ml stock solution with methanol up to 50 ml dilution.

Preparation of Test Solution:

Test solution was prepared by dissolving 25 mg sample of Dabigatran etexilate in 50 ml volumetric flask. The final test solution with 25 µg/ml concentration has been prepared by 2.5 ml stock solution with methanol up to 50 ml dilution.

Method Development and Optimization:

A method development and optimization is the most essential part of any chromatographic separation. Before develop rugged, simple and easy to use, reproduce and suitable UPLC method for the quantification of Dabigatran etexilate many trails were performed using column selection, mobile phase selection and appropriate pH. Best resolution, sharpness and shortest runtime was obtained in HSS C18 (50 X 2.1mm i.d., 1.8µm particle size) column and mobile phase with the constitution of buffer 0.1% Triethylamine in water having pH 5.0 ± 0.05 in glacial acetic acid. By the use of methanol as a diluents straight baseline observed during whole analysis. Total elution time of this method was only 3.0, so it is cheaper, less solvent consuming and time saving process. The method has been optimized in such a way that degrading impurity is not affected Dabigatran peak and also well resolves from product peak.

Forced degradation studies:

The degradation study was carried out to measure the stability indicating study and selectivity of the optimized method. The degradation study was performed to ensure that Dabigatran peak separate from different degradation products with high resolution. Force degradation study of Dabigatran etexilate was performed in different condition such as acidic, alkali and oxidative degradation. Dabigatran was highly sensitive towards stress conditions.

Acidic Degradation:
Acid Degradation study of Dabigataran etexilate was carried out at 250 µg/ml concentration in presence of 1 ml 0.1 M HCl. The reaction mass was kept at room temperature for 5 to 10 minute followed by neutralize with 1 ml 0.1 M NaOH solution. The resulting mass was diluted to make the final concentration 25 µg/ml. The above procedure was repeated with 0.5 M and 1.0 M HCl solution at 0 to 4 h time interval at room temperature.

Alkali Degradation:
Alkali Degradation study of Dabigataran etexilate was carried out at 250 µg/ml concentration in presence of 1 ml 0.1 M NaOH. The reaction mass was kept at room temperature for 5 to 10 minute followed by neutralize with 1 ml 0.1 M HCl solution. The resulting mass was diluted to make the final concentration 25 µg/ml. The above procedure was repeated with 0.5 M and 1.0 M NaOH solution at 0 to 4 h time interval at room temperature.

Oxidative Degradation:
Oxidizing degradation study of Dabigatran etexilate was performed at 250 µg/ml concentration by 3% H_2O_2 (v/v)**.** The resulting mass was diluted to make the final concentration 25 µg/ml. The above procedure was performed at room temperature for 0 to 4 h time interval. The degradation response of Dabigatran etexilate with different stress conditions were reported in Table 1 and Fig. (2 to 15)

Table 1 Evaluation data for force degradation study

Degradation Condition (At Room Temperature)	Mean Area	%Assay	%Drug decompose
Standard	620229	100.0	---
1 ml 0.1 N acid 0 h	534351	89.40	10.60
1 ml 0.1 N acid 4 h	143768	24.35	75.65
1 ml 0.5 N acid 0 h	504464	84.06	15.94
1 ml 0.5 N acid 4 h	97594	16.33	83.67
1 ml 1 N acid 0 h	475305	78.24	21.76
1 ml 1 N acid 4 h	28171	4.60	95.40
1 ml 0.1 N base 0 h	561014	93.48	6.52
1 ml 0.1 N base 4 h	12149	2.07	97.93
1 ml 0.5 N base 0 h	243726	41.98	58.02
1 ml 0.5 N base 4 h	4648	0.76	99.24
1 ml 1 N base 0 h	103499	17.32	82.68
1 ml 1 N base 4 h	1802	0.30	99.70
1 ml 3% H_2O_2 0 h	528619	90.28	9.72
1 ml 3% H_2O_2 4 h	220926	36.52	63.48

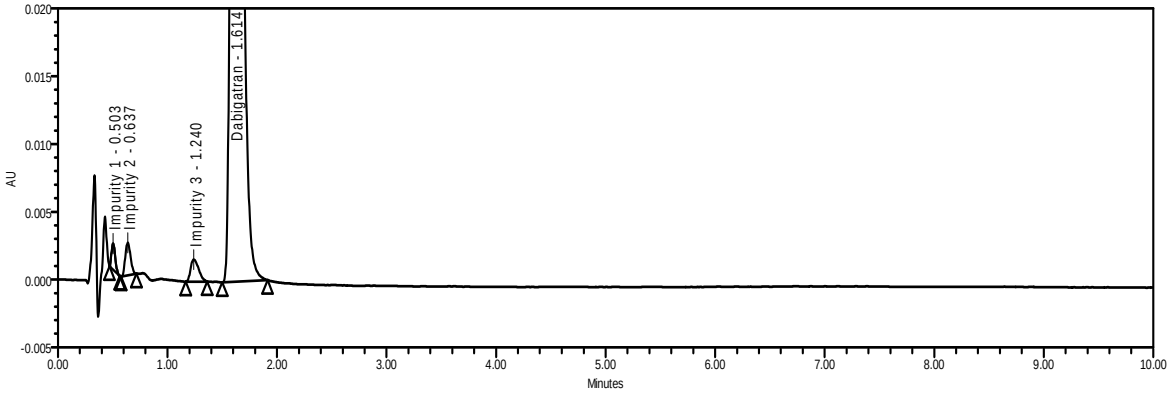

Fig. 2: Chromatograph of Dabigatran etexilate under acid stress condition, 1 ml 0.1 N acid at room temperature for 0 h time interval

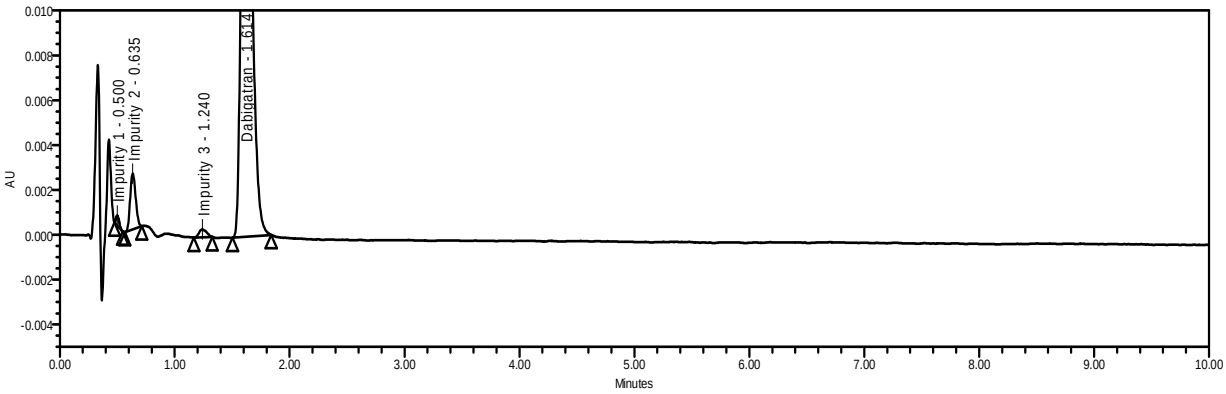

Fig. 3: Chromatograph of Dabigatran etexilate under acid stress condition, 1 ml 0.1 N acid at room temperature for 4 h time interval

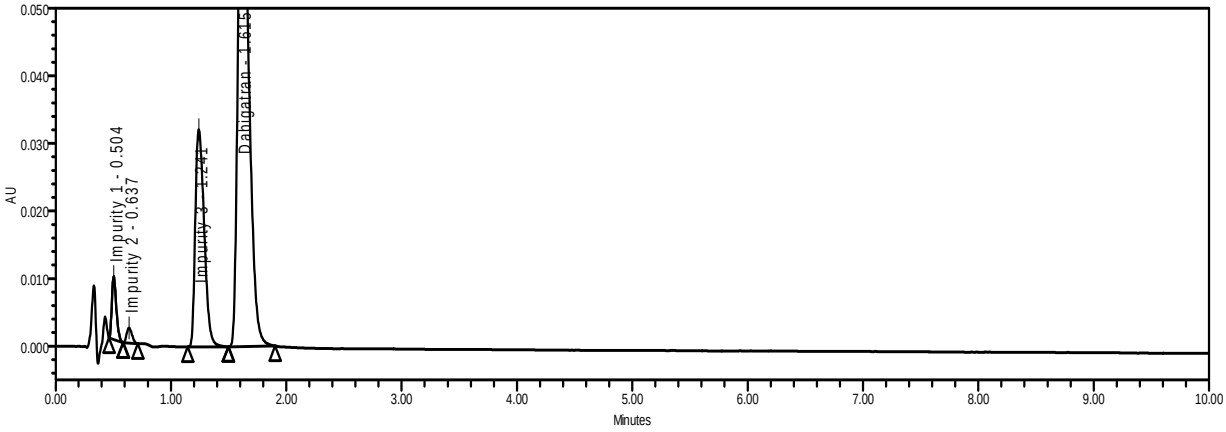

Fig. 4: Chromatograph of Dabigatran etexilate under acid stress condition, 1 ml 0.5 N acid at room temperature for 0 h time interval

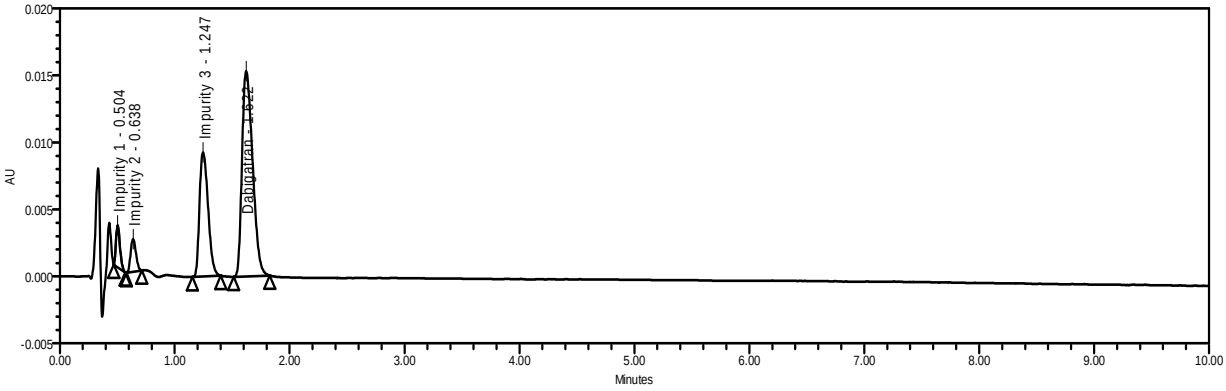

Fig. 5: Chromatograph of Dabigatran etexilate under acid stress condition, 1 ml 0.5 N acid at room temperature for 4 h time interval

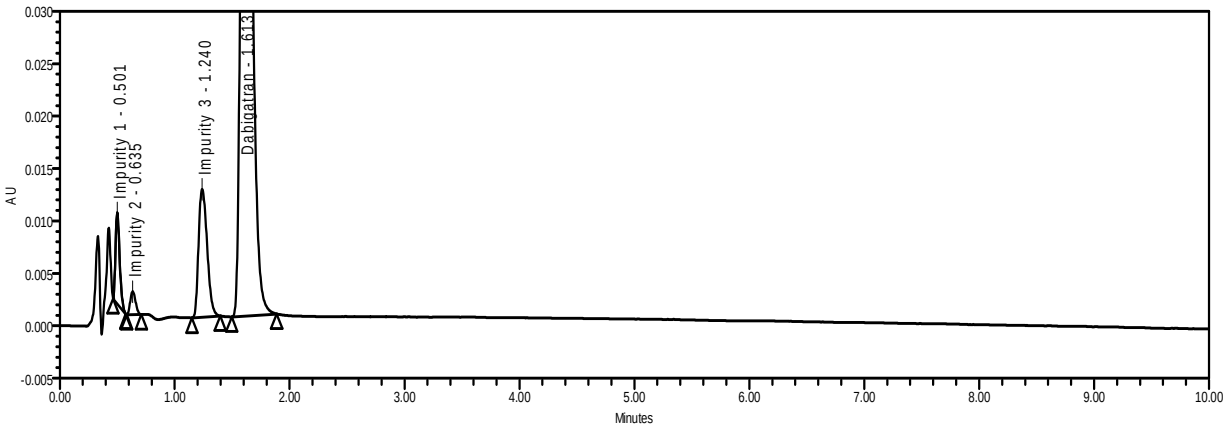

Fig. 6: Chromatograph of Dabigatran etexilate under acid stress condition, 1 ml 1 N acid at room temperature for 0 h time interval

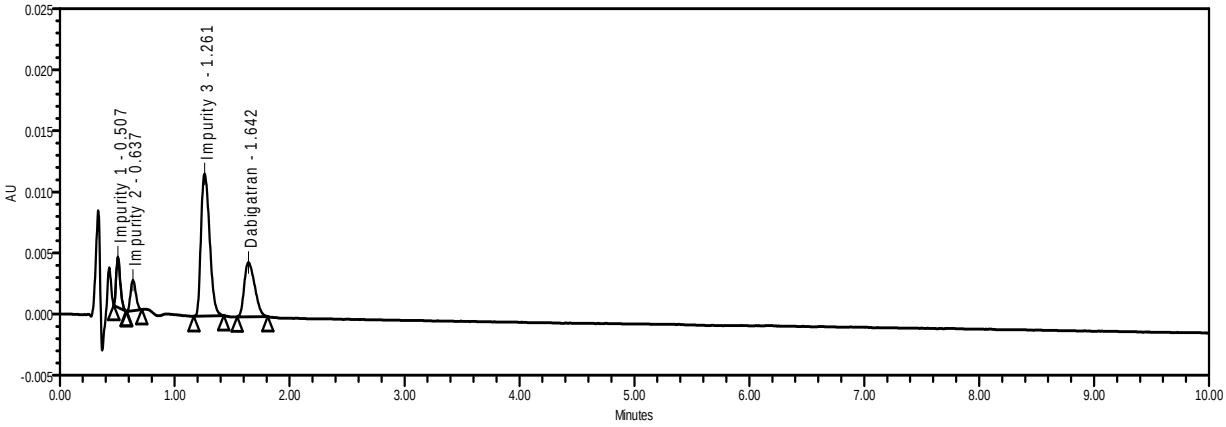

Fig. 7: Chromatograph of Dabigatran etexilate under acid stress condition, 1 ml 1 N acid at room temperature for 4 h time interval

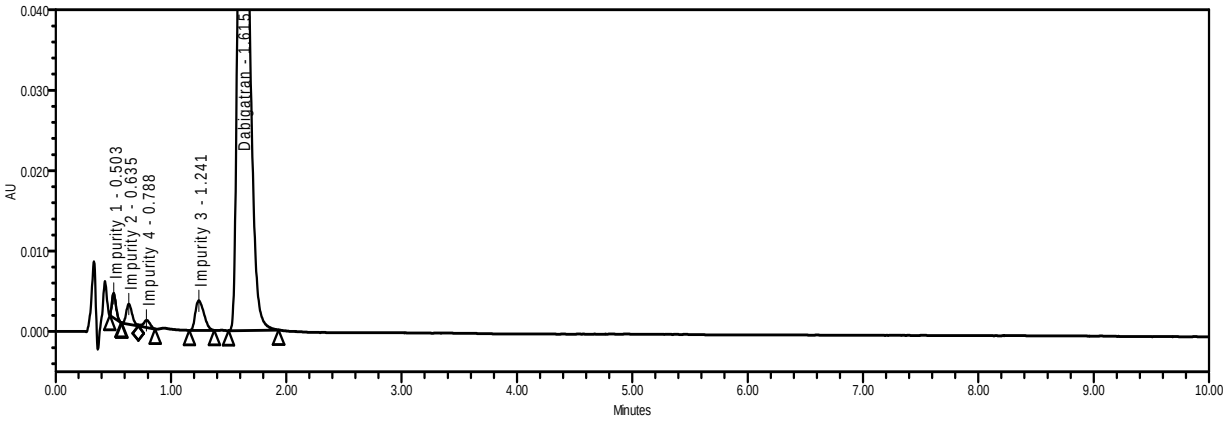

Fig. 8: Chromatograph of Dabigatran etexilate under Basic stress condition, 1 ml 0.1 N base at room temperature for 0 h time interval

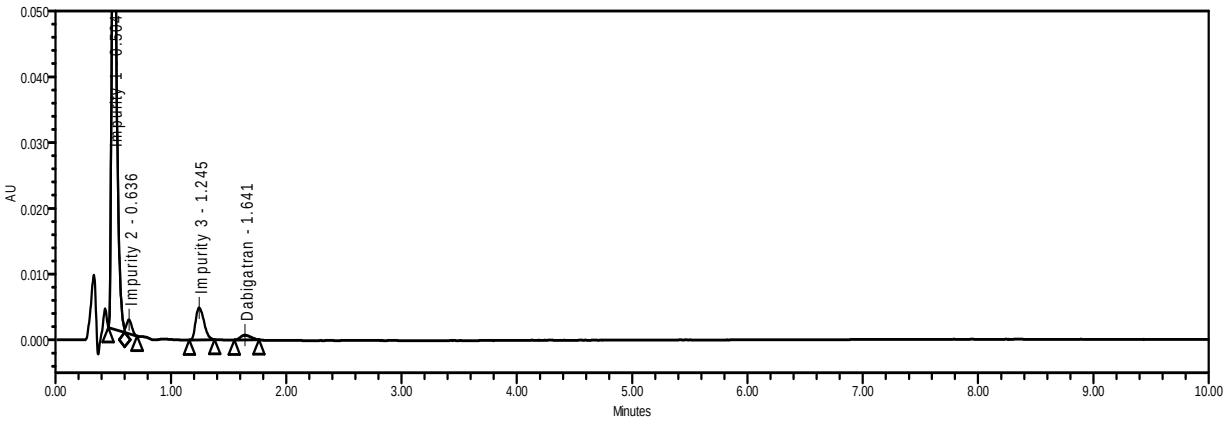

Fig. 9: Chromatograph of Dabigatran etexilate under Basic stress condition, 1 ml 0.1 N base at room temperature for 4 h time interval

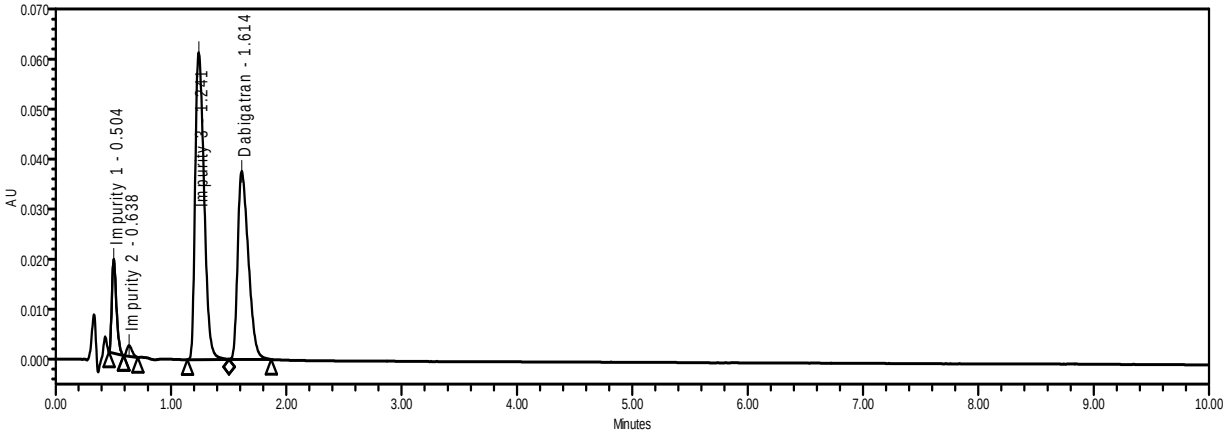

Fig. 10: Chromatograph of Dabigatran etexilate under Basic stress condition, 1 ml 0.5 N base at room temperature for 0 h time interval

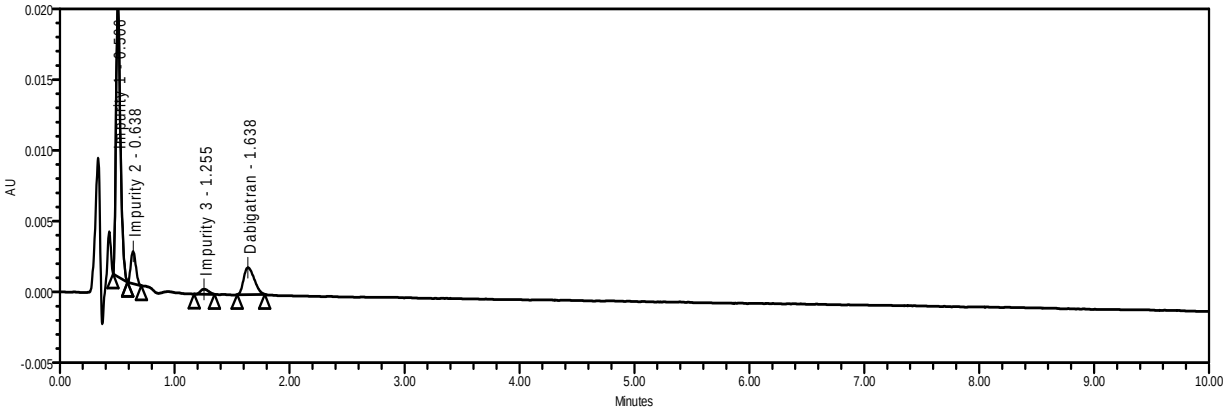

Fig. 11: Chromatograph of Dabigatran etexilate under Basic stress condition, 1 ml 0.5 N base at room temperature for 4 h time interval

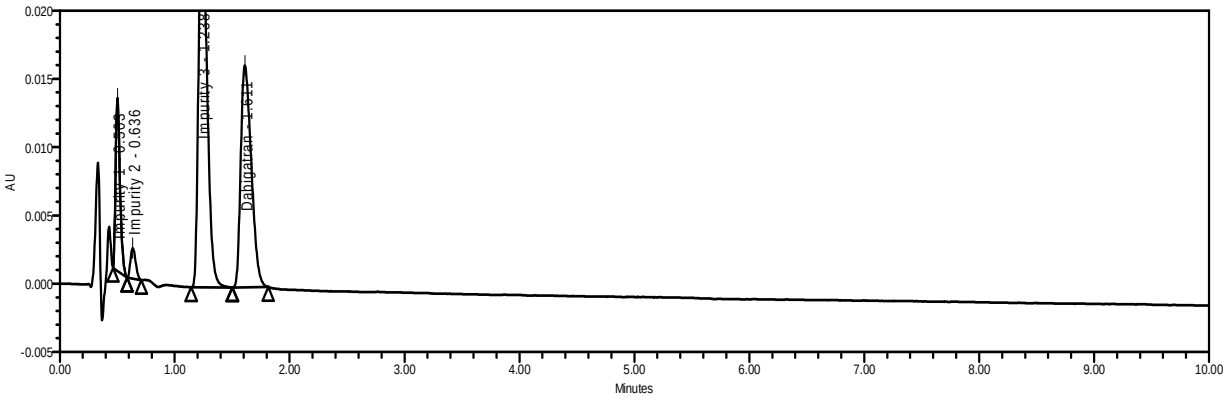

Fig. 12: Chromatograph of Dabigatran etexilate under Basic stress condition, 1 ml 1 N base at room temperature for 0 h time interval

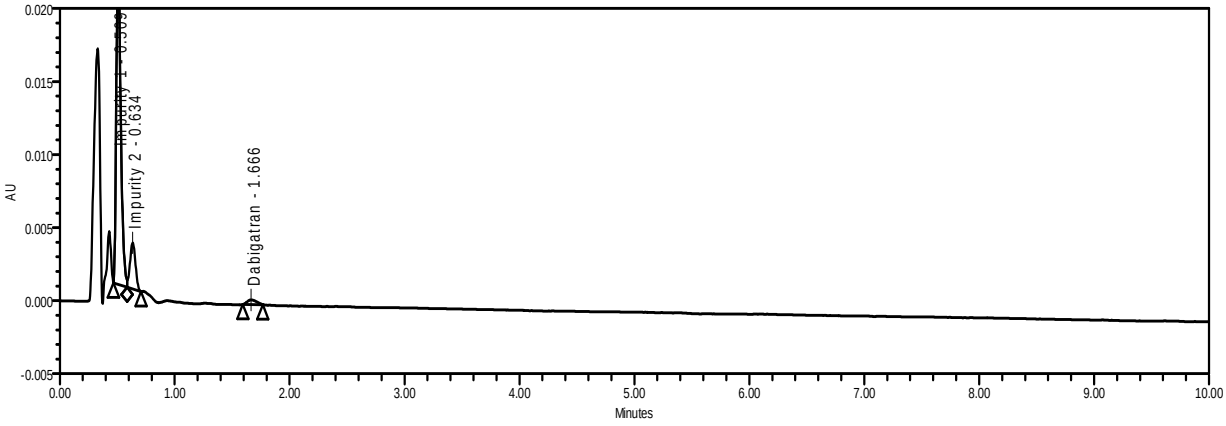

Fig. 13: Chromatograph of Dabigatran etexilate under Basic stress condition, 1 ml 1 N base at room temperature for 4 h time interval

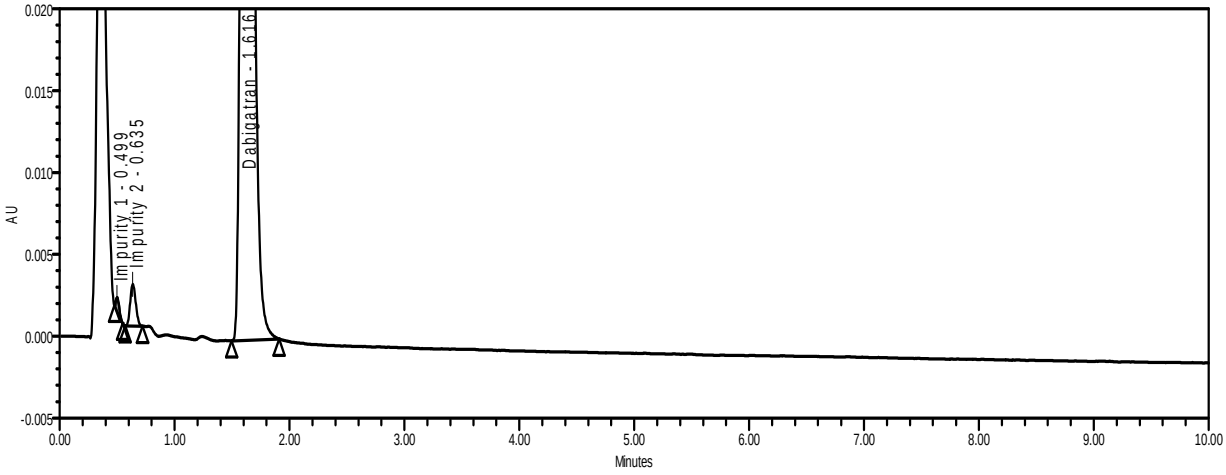

Fig. 14: Chromatograph of Dabigatran etexilate under oxidative stress condition, 1 ml 3% H_2O_2 at room temperature for 0 h time interval

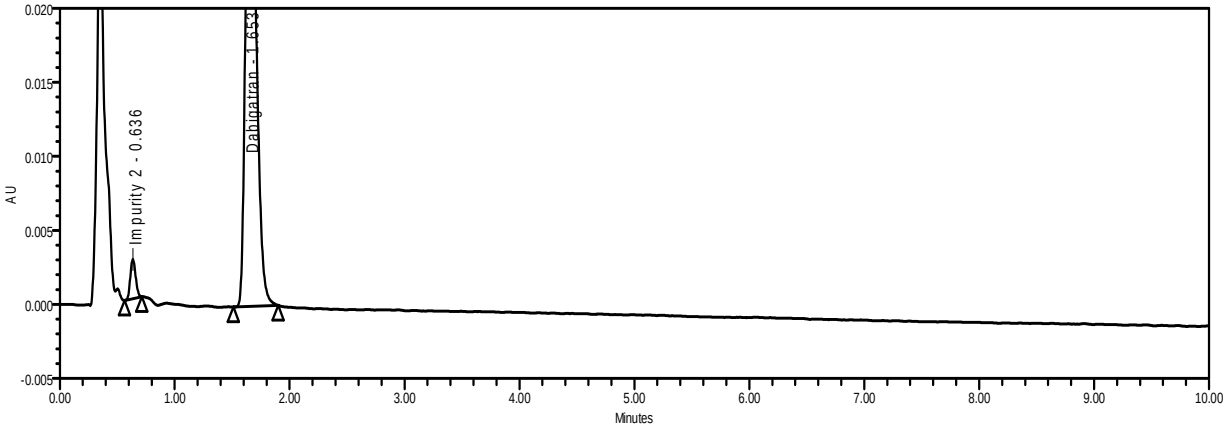

Fig. 15: Chromatograph of Dabigatran etexilate under oxidative stress condition, 1 ml 3% H_2O_2 at room temperature for 4 h time interval

Method Validation:

The proposed method was validated as per ICH guideline. Method validation was performed using parameters like Specificity, Accuracy, Precision, Linearity, Limit of detection (LOD), limit of quantification (LOQ), Solution stability, Robustness and System suitability.

Specificity:

The specificity of the method was evaluated to ensure that there was no any interference of diluents, mobile phase and degradation products.

Accuracy:

For the determination of reliability and suitability of the developed and optimized method, accuracy experiments were carried out. Accuracy is the measurement of exactness of an analytical method and closeness of analyte value which is determined from drug sample and the value that is accepted either as a conventional, true value or accepted reference value. Accuracy of Dabigatran etexilate was measured by three accuracy levels 50%, 100% and 150% of the analyte concentration (25 µg/ml). Each level of accuracy was prepared in three sets and each set was injected in duplicate. The data for the accuracy study was evaluated in Table 2.

Table 2 Summery of accuracy study

Accuracy Level	Set No.	Drug Amount added (µg/ml)	Drug Amount found (µg/ml)	%Recovery	Mean %Recovery	% RSD
50%	1	12.60	12.73	101.06	100.70	0.35
	2	12.70	12.75	100.35		
	3	12.60	12.69	100.70		
100%	1	25.30	25.51	100.82	100.33	0.53
	2	25.40	25.51	100.42		
	3	25.50	25.44	99.76		
150%	1	36.90	36.79	99.70	99.68	0.44
	2	37.20	36.92	99.24		
	3	37.08	37.12	100.11		

Precision:

The precision of the developed and optimized method was evaluated by repeatability and intermediate precision. Repeatability of the method precision was performed by injecting six sets of Dabigatran etexilate sample and calculates the %RSD. The %RSD must be less than 2%. Intermediate precision was performed by different analyst and different instrument, performing same analysis on different days. Further it is supported by the calculation of %RSD of Dabigatran etexilate peak for different sets. The data related to precision was reported in Table 3.

Table 3 Summery of precision study

Study	Precision Set No.	%Assay	Mean %Assay	Std.dev.	%RSD
Method Precision	1	100.82	99.99	0.66	0.66
	2	99.96			
	3	99.47			
	4	99.2			
	5	100.73			
	6	99.76			
Intermediate Precision	1	99.68	99.93	0.30	0.30
	2	99.92			
	3	100.47			
	4	99.72			
	5	100.04			
	6	99.77			
Overall	Mean	99.96			
	Std. dev.	0.04			
	%RSD	0.04			

Linearity:
Linearity for the analytical method was performed by using nine level concentrations from the range 5 to 45 µg/ml. Each level of concentration was injected in duplicate and calculate the concentration using calibration curve of peak area vs. concentrations. The method was linear in all above concentration range and regression coefficient was found 0.998.

Limit of detection (LOD) and Limit of quantification (LOQ):
LOD was expressed by establishing the lower level at which analyte can be reliably detected. LOQ was considered as the lowest concentration of the Dabigatran etexilate reference standard that can be reproducibly detected with acceptable criteria (%RSD<2). LOD and LOQ were determined by signal-to-noise ratio (S/N). A signal-to-noise ratio between 3:1 is generally considered acceptable for estimating the detection limit. For quantification limit signal-to-noise ratio is 10:1. LOD and LOQ for Dabigatran etexilate were determined by injecting a series of dilute solution of known concentration of Dabigatran etexilate sample. The reproducibility of LOQ was measured by injecting six replicate injections of lowest concentration of analyzed standard. The LOD and LOQ were found 0.05 and 0.5 µg/ml respectively.

Solution Stability:
Solution stability was performed by keeping the sample solutions at initial to 4 h time interval at room temperature in presence of light. The sample solutions of different time interval was injected and compared with freshly prepared standard and test solution. The summary of solution stability was reported in Table 4.

Table 4 Solution stability study

Solution stability samples	Mean Area	%Assay
Initial	668167	99.75
4 h	661656	99.34
8 h	662115	99.57
12 h	664017	99.45
16 h	655157	99.16
20 h	658993	99.10
24 h	658244	98.99
36 h	654147	98.77
48 h	653918	98.73

Robustness:
The Robustness is an analytical procedure which is measured by small but deliberate change in analytical parameters like Flow rate, Mobile phase composition, pH value of mobile phase, use different types of analytical columns and column oven temperature. Robustness is providing its reliability during normal usage. The variables evaluated in the study were Column oven temperature (30°C ± 5°C), Flow rate (0.35 ± 0.01 ml/min) and Mobile phase composition was (Buffer: Acetonitrile = 49:51 and 51:49, v/v). The data related to robustness was depicted in Table 5.

Table 5 Evaluation data for robustness study

Robustness parameters	% Assay	RT, min	System suitability parameters	
			USP Tailing	Theoretical Plates
Flow rate 0.34 ml/min	99.73	1.62	1.40	2957
Flow rate 0.36 ml/min	99.57	1.54	1.39	2926
Buffer: Acetonitrile (49:51 v/v)	99.79	1.48	1.37	3120
Buffer: Acetonitrile (51:49 v/v)	99.89	1.62	1.38	3025
Column oven temperature 25°C	99.84	1.56	1.40	2980
Column oven temperature 35°C	99.44	1.60	1.39	3115

System suitability:

The System suitability test was performed to measure the resolution and reproducibility of the system. The system suitability of chromatographic system was carried out before each validation parameters. Five replicate injection of standard preparation and duplicate injection of sample were injected and system suitability parameters like Theoretical plates, USP tailing and %RSD of peak area were calculated. System suitability data were reported in Table 6.

Table 6 system suitability criteria for Dabigatran etexilate

System suitability parameters	Acceptance criteria	Observed value
USP Tailing	NMT 2.0	1.3
% Relative standard deviation	NMT 2.0%	0.2
Theoretical plates	NLT 2000	3588

**NMT: NOT MORE THAN*
**NLT: NOT LESS THAN*

RESULTS AND DISCUSSION

UPLC method for analysis of pharmaceutical drug substances is more accurate, precise, and better resolution, chiper and time saving process then HPLC system. In UPLC columns have very small particle size so they give better resolution and high efficiency. The runtime in UPLC method was found to be less as compare to HPLC method. The proposed method was well developed and validated on UPLC for quantitative analysis of Dabigatran etexilate with degradation study under different stress conditions.

Sharp peak and better resolution for Dabigatran etexilate was obtained by using HSS C18 (50 x 2.1 mm) with 1.8 µm particle size column, Dabigatran peak was obtained at 1.5 ± 0.05 during 3.0 min over all run time which was proved that it is time saving and less solvent consumed method i.e. cost effective method. The number of theoretical plate was NLT 2000 and USP tailing was NMT 2.0 which indicates excellent column performance for Dabigatran etexilate. The chromatograph of Dabigatran etexilate of standard and sample were shown in Fig. 16 and 17 respectively.

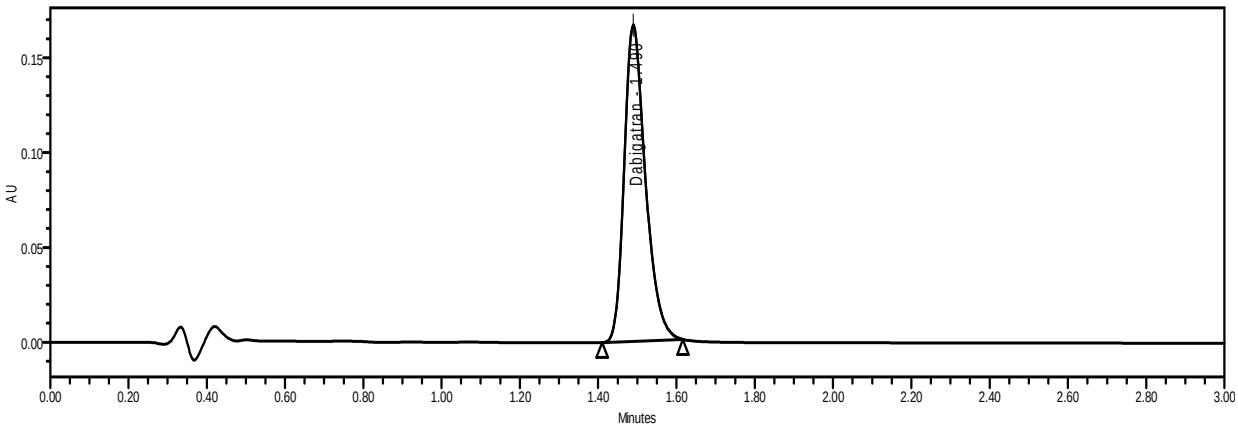

Fig. 16: Standard Chromatograph of Dabigatran etexilate (25 µg/ml)

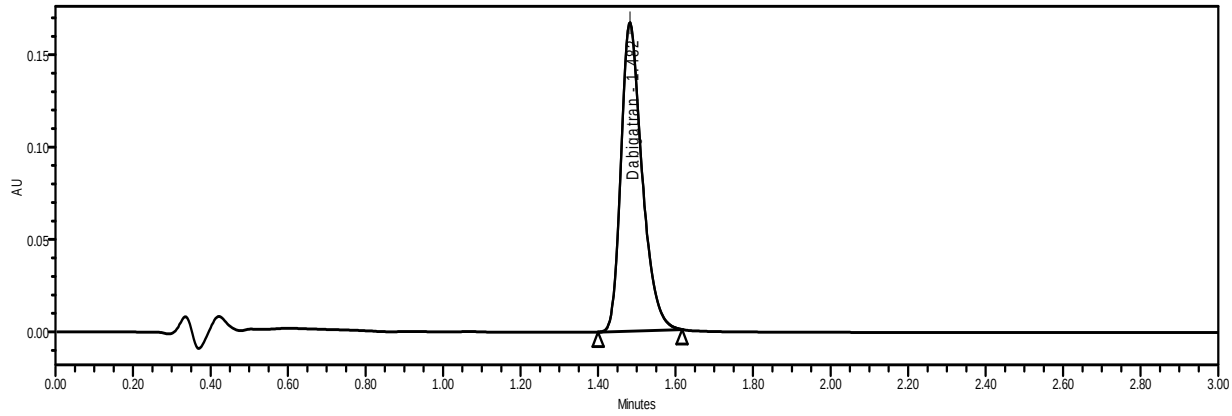

Fig. 17: Sample Chromatograph of Dabigatran etexilate (25 µg/ml)

The linearity of Dabigatran etexilate was obtained from LOQ to 180% of the actual concentration (25 µg/ml). Which was calibrated in the range of 0.5 to 45 µg/ml concentration and linear regression equation was computed as Y = 12622x + 24016, r^2 = 0.998 (Fig. 18). The evaluation data for linearity study was reported in Table 7.

Table 7 Summary of linearity concentration evaluation of Dabigtran etexilate

Linearity Level	Concentration (µg/ml)	Mean Area
Level 1 (20%)	5.04	143258
Level 2 (40%)	10.08	267745
Level 3 (60%)	15.12	402670
Level 4 (80%)	20.16	541834
Level 5 (100%)	25.2	642521
Level 6 (120%)	30.24	803845
Level 7 (140%)	35.28	925672
Level 8 (160%)	40.32	1032906
Level 9 (180%)	45.36	1135727

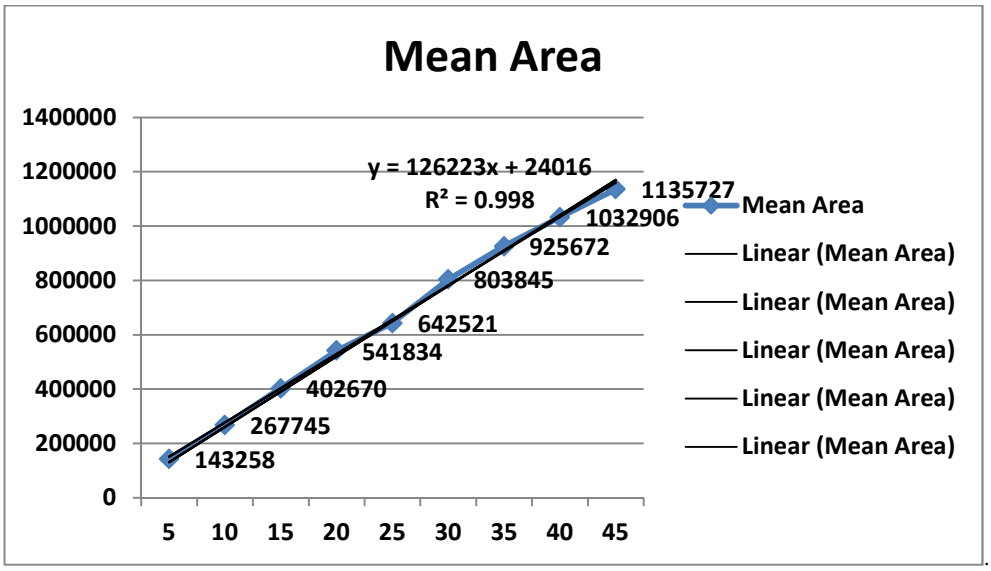

Fig. 18: Linearity curve of Dabigatran etexilate

The % recovery of the sample was found in the range of 99.68 to 100.70, while mean recovery and % RSD were 100.24 and 0.44 respectively. The precision value for each test prepared were found in the range 99.0 – 101.0 and % RSD related to precision was less than 2.0 which was further conformation for highly precise method.

CONCLUSION

A simple, accurate, precise and time saving Ultra high performance Liquid Chromatographic method was

developed, optimized and validated as per ICH guideline. The overall run time was only 3 min and Dabigatran peak was observed at 1.5 min. i.e. it is very coast effective method. This method is very sensitive towards force degradation and all the degradation products are well separated from the peak of interest. A method consist every degradation parameters and so we found actual storage condition of Dabigatran etexilate drug substance.

Acknowledgements
Authors are thankful to Centre for Excellence, NFDD complex and Department of Chemistry, Saurashtra University Rajkot to provide such facilities and also thankful to Amney Pharmaceutical Ltd, Ahmedabad – Bavla highway, Ahmedabad for providing samples for above research work.

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