IJPSR (2021), Volume 12, Issue 3 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 23 March 2020; received in revised form, 25 June 2020; accepted, 30 June 2020; published 01 March 2021

NOVEL METHOD FOR SEPARATION AND QUANTIFICATION OF POTENTIAL IMPURITIES BY RP-HPLC FROM KSM STAGE TO API STAGE OF DABIGATRAN MESYLATE

T. Naga Jhansi [*1, 2], R. Nagaraju [1, 2], D. Jaya Deep Kumar [1] and G. Nageswara Rao [2]

Department of Analytical Research and Development [1], Dr. Reddy's Laboratories Ltd., Srikakulam - 532407, Andhra Pradesh, India.
Department of Inorganic and Analytical Chemistry [2], Andhra University, Visakhapatnam - 530003, Andhra Pradesh, India.

Keywords:

Dabigatran, RP-HPLC, Potential impurities, Forced degradation and validation

Correspondence to Author:
T. Naga Jhansi

Research Associate,
Department of Analytical Research and Development, Dr. Reddy's Laboratories Ltd., Srikakulam - 532407, Andhra Pradesh, India.

E-mail: jhansireddyau@gmail.com

ABSTRACT: Dabigatran mesylate is a novel oral anticoagulant that works by blocking the clotting protein thrombin. The present work describes the separation and quantification of potential impurities in a single HPLC method, which are generated from raw materials to API synthesis. This method is capable of separating and quantifying the fourteen impurities, produced from the intermediate stages to final drug substance stage of dabigatran within 40 min of run time. Column: Poroshell 120, EC C-18, 150 mm × 4.6 mm, 2.7 µ; Buffer: 2.04 g of Potassium dihydrogen phosphate in a beaker, add 1500 mL of water and dissolve, to this add 1.5 ml Triethylamine (TEA) and 1 ml of Phosphoric acid; Diluent: water and acetonitrile in the ratio 20:80% v/v; The flow rate: 0.7 mL/min; Column temperature: 40 °C. Wavelength: 230 nm; The drug substance was subjected to stress studies such as hydrolysis, oxidation and thermal degradation and considerable degradation was observed in acidic hydrolysis and oxidative stress conditions. The formed degradation products were well-resolved from the dabigatran drug substance and its related impurities. The validated method produced good results of precision, linearity, accuracy, robustness and ruggedness. The proposed method was found to be suitable precise, sensitive and accurate for the quantitative determination of related impurities in the samples of Dabigatran mesylate drug substance.

INTRODUCTION: Dabigatran mesylate is a potent, non-peptidic small molecule that specifically and reversibly inhibits both free and clot bound thrombin. It has been approved for the prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation by US Food and Drug Administration in October 2010 and by European Medicines Agency (EMA) in August 2011.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(3).1762-79

This article can be accessed online on www.ijpsr.com

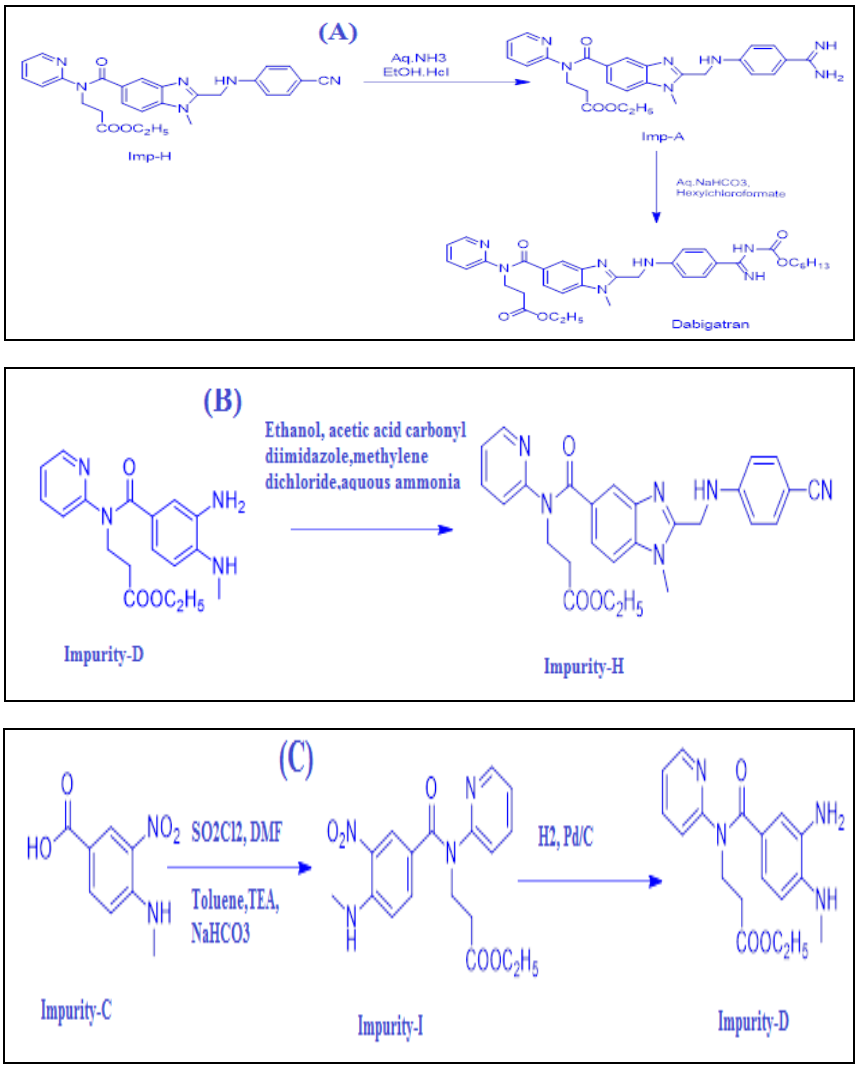
DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(3).1762-79

EMA also approved it in 2008 for prophylaxis of thromboembolism in patients undergoing total knee or hip replacement (EMEA, 2008). Dabigatran mesylate capsule is marketed under the trade name Pradaxa in the United States, Australia, and European countries [1-3].

Oral administration of Dabigatran mesylate produces predictable pharmacodynamics and has been clinically developed in various indications using fixed-dosing without the need for routine coagulation monitoring or dose adjustment. Anticoagulant treatment reduces the incidence of death and cardioembolic events. Since thrombin plays a key role in the formation of fibrin and is very important for blood coagulation and platelet activation, it represents a prime target for the

development of anticoagulant agents for the prevention and treatment of thromboembolic disorders [4-6]. Several research papers have been reported in the literature for the determination of dabigatran and its impurities, such as Bernardi *et al.*, 2013a, Bernardi *et al.*, 2013b, Damle and Bagwe, 2014, Geetharam *et al.*, 2014, Pani Kumar *et al.*, 2015, Khan *et al.*, 2014, Reddy and Rao, 2014 and these papers were limited to the assay of alone dabigatran from its few impurities [7-11]. But this proposed HPLC method was suitable to monitor stage-wise by-products and raw materials during the synthesis of dabigatran mesylate. Monitoring and control of by-products or impurities at each stage of synthesis is required to get a final pure form of dabigatran as per predefined specifications. The reported related substance methods were not suitable to monitor stage-wise by-products during the synthesis of dabigatran and were only restricted to some of the related impurities [12-16].

To the best of our knowledge, a complete validated HPLC method for the determination of impurities at different stages of synthesis, i.e., raw materials, by-products, and degradants of dabigatran in drug substances, is not reported till date. During the analysis of laboratory batches from synthetic process development of dabigatran, the impurity-A to impurity-N (**Fig. 1-A, B, C, D, E, F, G, H, I, J, K, L, M,** and **N)** were identified as potential monitoring impurities to ensure the quality of active pharma ingredient (API) by controlling them less than 0.1 % as per ICH The present manuscript describes, development of an RP-HPLC method for the separation and determination of Dabigatran potential related impurities, *i.e.*, raw materials, by-products and degradants of mentioned synthetic procedure (namely Impurities-A, -B, -C, -D, -E, -F, -G, -H, -I, -J, -K, -L, -M and -N) and validation as per ICH guidelines [16-20]. The formation of different potential impurities is shown in **Fig. 1.**



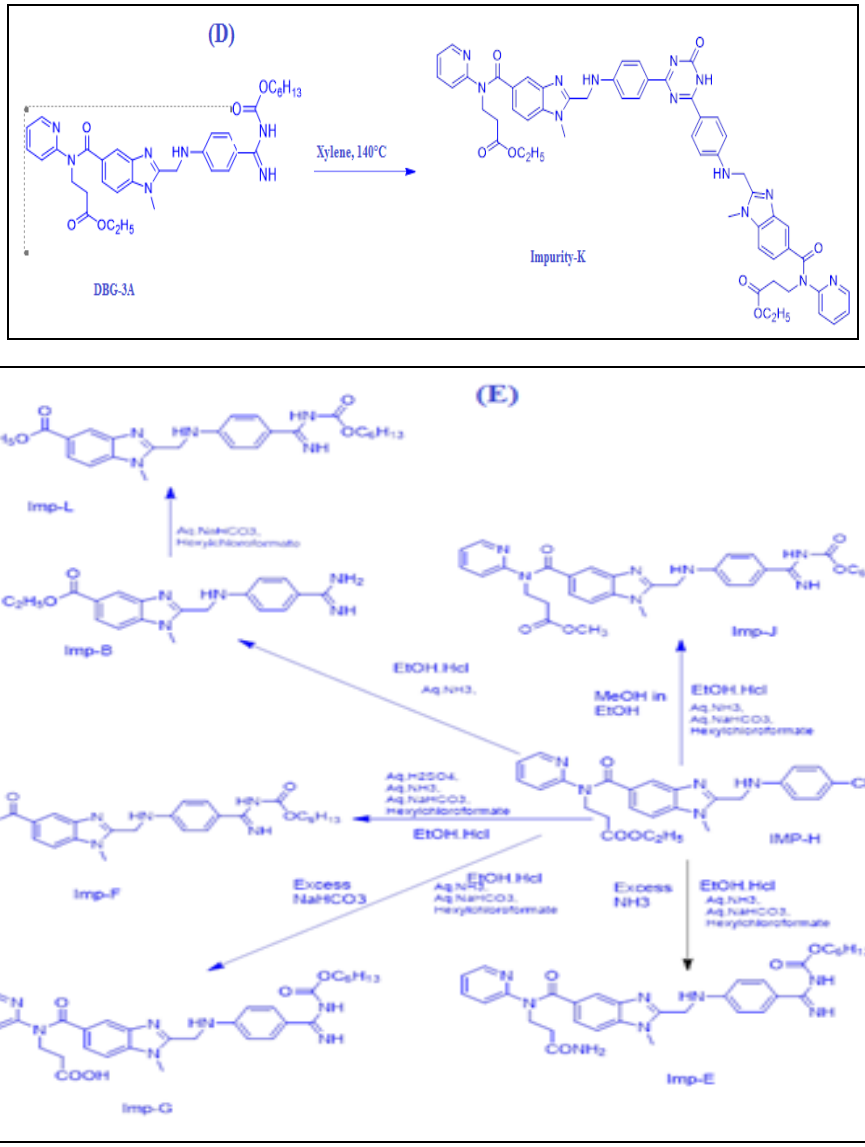
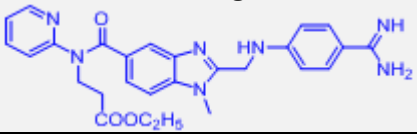


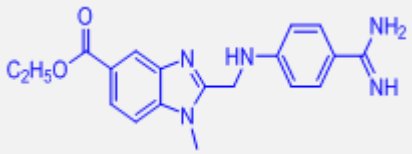
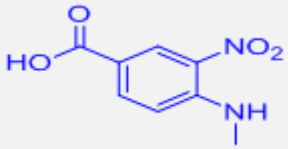
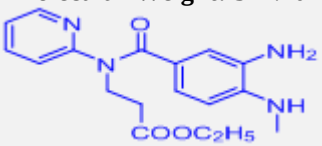
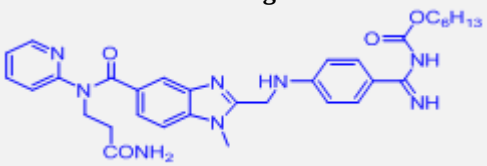
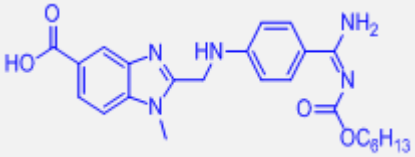
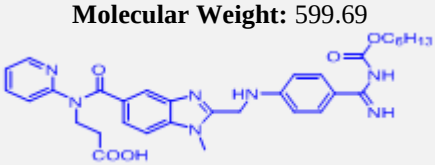
FIG. 1: FORMATION OF IMPURITIES AT DIFFERENT STAGES OF DABIGATRAN (A) PREPARATION OF DBG2 (IMPURITY-A) INTERMEDIATE FORM CBA (IMPURITY-H) RAW MATERIAL. (B) PREPARATION OF CBA (IMPURITY-H) RAW MATERIAL FROM CBA3 RAW MATERIAL (IMPURITY-D). (C) PREPARATION OF (CBA3 RAW MATERIAL) IMPURITY-D FORM CBA2 (IMPURITY-C). (D) PREPARATION OF IMPURITY-K FROM DBG3A (E) FORMATION OF OTHER IMPURITIES FROM IMPURITY-H

MATERIALS AND METHOD:

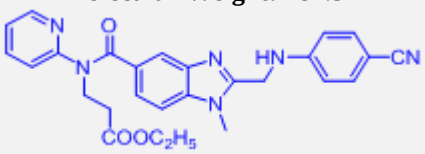
Chemicals & Instrument: Potassium dihydrogen phosphate, orthophosphoric acid, Tri ethyl amine (AR grade); Acetonitrile (HPLC grade); Water (Milli-Q grade). Agilent HPLC instrument with PDA detector; Poroshell 120, EC C-18,150 mm × 4.6 mm, 2.7 µ column; Data was processed through empower-3 software.

TABLE 1: THE STRUCTURE OF DABIGATRAN AND ITS IMPURITIES WERE CAPTURED IN TABLE 1

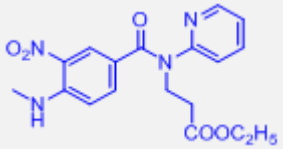
S. no.	Impurity Details and Structure
1	**Chemical name:** Ethyl 3-(2-(((4-carbamimidoyl phenyl) amino) methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo [d] imidazole-5-carboxamido) propanoate. **Identification Name:** Impurity-A **Chemical Formula:** $C_{27}H_{29}N_7O_3$ **Molecular Weight:** 499.58 

2	**Chemical name:** Ethyl 2-(((4-carbamimidoyl phenyl) amino) methyl)-1-methyl-1H-benzo[d]imidazole-5-carboxylate. **Identification Name:** Impurity-B **Chemical Formula:** $C_{19}H_{21}N_5O_2$ **Molecular Weight:** 351.41 
3	**Chemical name:** 4-(Methylamino)-3-nitro benzoic acid **Identification Name:** Impurity-C **Chemical Formula:** $C_8H_8N_2O_4$ **Molecular Weight:** 196.16 
4	**Chemical name:** Ethyl-3-(3-Amino-4-(methyl amino)-N-Pyridine-2-yl-) benzamido propanoate. **Identification Name:** Impurity-D **Chemical Formula:** $C_{18}H_{22}N_4O_3$ **Molecular Weight:** 342.40 
5	**Chemical name:** Hexyl ((4-(((5-((3-amino-3-oxopropyl) (pyridin-2-yl) carbamoyl)-1-methyl-1H-benzo [d] imidazol-2-yl) methyl) amino) phenyl)(imino) methyl) carbamate. **Identification Name:** Impurity-E **Chemical Formula:** $C_{32}H_{38}N_8O_4$ **Molecular Weight:** 598.71 
6	**Chemical name:** 2-(((4-(N-((hexyloxy) carbonyl) carbamimidoyl) phenyl) amino) methyl)-1-methyl-1H-benzo [d] imidazole-5-carboxylic acid. **Identification Name:** Impurity-F **Chemical Formula:** $C_{24}H_{29}N_5O_4$ **Molecular Weight:** 451.53 
7	**Chemical name:** 3-(2-(((4-(N-((hexyloxy) carbonyl) carbamimidoyl) phenyl) amino) methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo[d] imidazole-5-carboxamido) propanoic acid **Identification Name:** Impurity-G **Chemical Formula:** $C_{32}H_{37}N_7O_5$ **Molecular Weight:** 599.69 
8	**Chemical name:** Ethyl 3-(2-(((4-cyanophenyl) amino) methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo [d]imidazole-5-carboxamido) propanoate.

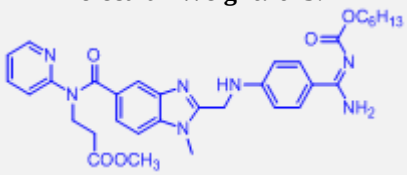
Identification Name: Impurity-H
Chemical Formula: $C_{27}H_{26}N_6O_3$
Molecular Weight: 482.54



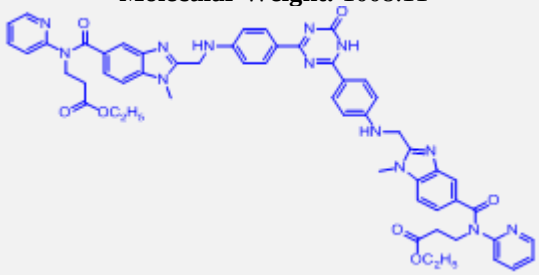
9 **Chemical name:** Ethyl-3-(3-nitro-4-(methyl amino)benzyl)pyridine-2-yl-) amino)propionate
Identification Name: Impurity-I
Chemical Formula: $C_{18}H_{20}N_4O_5$
Molecular Weight: 372.38



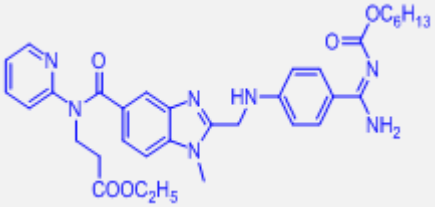
10 **Chemical name:** Methyl 3-(2-(((4-(N-((hexyloxy) carbonyl) carbamimidoyl) phenyl) amino) methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo [d]imidazole-5-carboxamido) propanoate.
Identification Name: Impurity-J
Chemical Formula: $C_{33}H_{39}N_7O_5$
Molecular Weight: 613.72



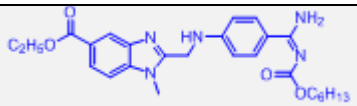
11 **Chemical name:** Diethyl 3,3'-((2,2'-((((6-oxo-1,6-dihydro-1,3,5-triazine-2,4-diyl) bis(4,1-phenylene)) bis (azanediyl)) bis (methylene))bis(1-methyl-1H-benzo [d] imidazole-2,5-diyl-5-carbonyl)) bis (pyridin-2-ylazanediyl)) dipropionate
Identification Name: Impurity-K
Chemical Formula: $C_{55}H_{53}N_{13}O_7$
Molecular Weight: 1008.11



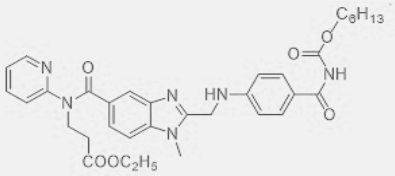
12 **Chemical name:** Ethyl 3-(2-(((4-(N-((hexyloxy) carbonyl) carbamimidoyl) phenyl) amino) methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo [d] imidazole-5-carboxamido) propanoate.
Identification Name: Dabigatran
Chemical Formula: $C_{34}H_{41}N_7O_5$
Molecular Weight: 627.75



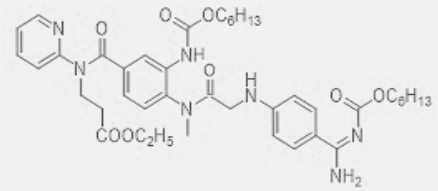
13 **Chemical name:** Ethyl 2-(((4-(N-((hexyloxy) carbonyl) carbamimidoyl) phenyl) amino)methyl)-1-methyl-1H-benzo[d]imidazole-5-carboxylate
Identification Name: Impurity-L
Chemical Formula: $C_{26}H_{33}N_5O_4$
Molecular Weight: 479.58



14 **Chemical name:** Ethyl 3-(2-(((4-(((hexyloxy) carbonyl) carbamoyl) phenyl) amino) methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo [d] imidazole-5-carboxamido) propanoate.
Identification Name: Impurity-M
Chemical Formula: $C_{34}H_{40}N_6O_6$
Molecular Weight: 628.73



15 **Chemical name:** Ethyl 3-(3-(((hexyloxy) carbonyl) amino)-4-(2-((4-(N-((hexyloxy) carbonyl) carbamimidoyl) phenyl) amino)-N-methylacetamido)-N-(pyridin-2-yl) benzamido) propanoate.
Identification Name: Impurity-N
Chemical Formula: $C_{41}H_{55}N_7O_8$
Molecular Weight: 773.93



Chromatographic Conditions: The analysis was carried out on column Poroshell 120, EC C-18, 150 mm x 4.6 mm, 2.7 µm particle size, buffer: 2.04 g of potassium dihydrogen phosphate into 1500 mL of water, add 1.5 ml triethylamine and 1 ml of phosphoric acid.

Mobile Phase A: buffer and acetonitrile in the ratio of 95: 5 v/v; mobile phase B: buffer and Acetonitrile in the ratio of 40: 60 v/v. Diluent: acetonitrile: water in the ratio of 800:200 (v/v); flow rate: 0.7 mL/min; column temperature: 40 °C; wavelength: 230 nm; injection volume: 10 µL; sample concentration: 0.5 mg/mL. Gradient program T/%B: 0/35, 5/35, 30/65, 35/90, 40/90. For the preparation of impurities H and L stock solutions, dimethylformamide was added 0.5 mL initially and then made up to the required volume with common diluent.

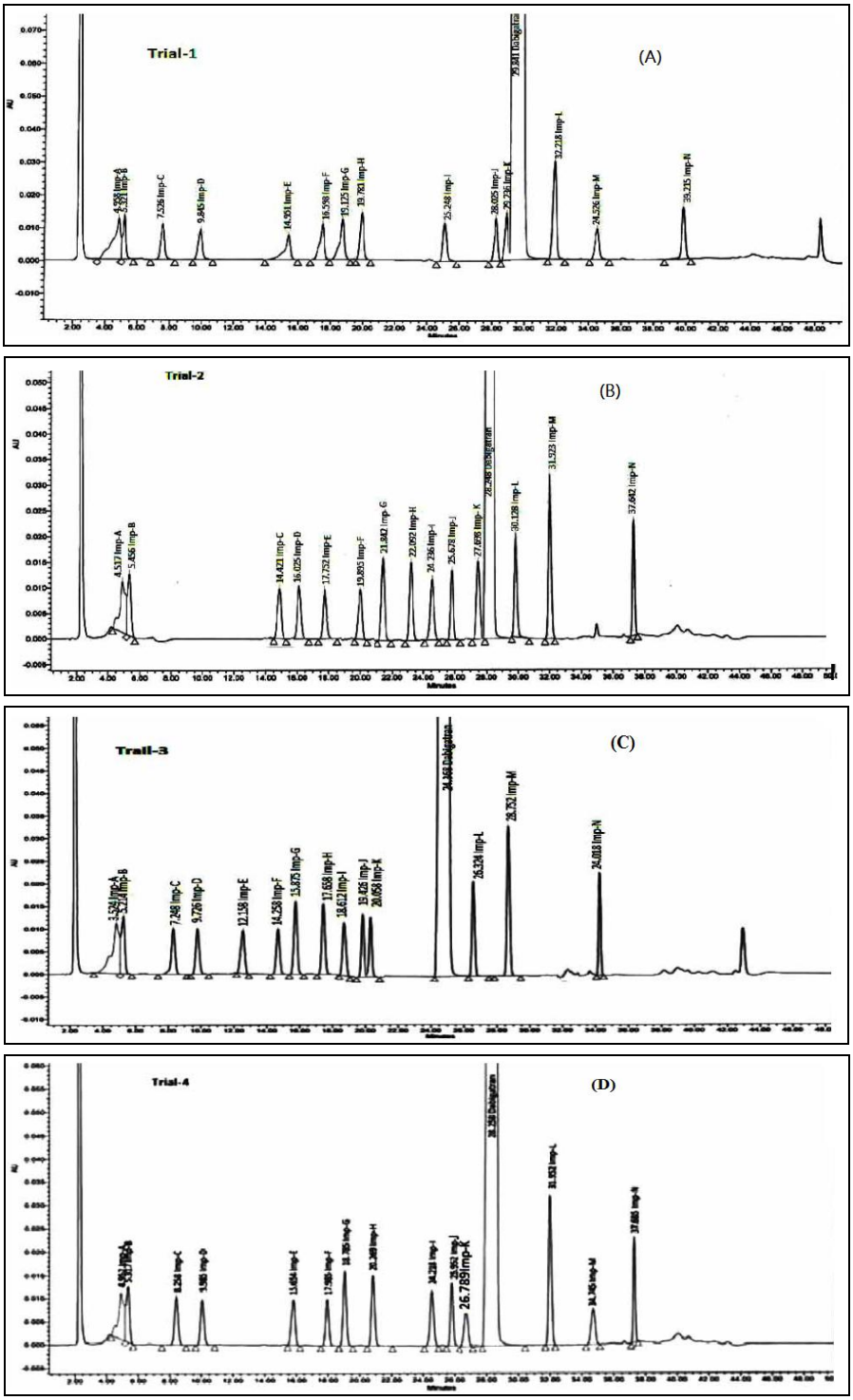
Standard and Sample Preparation: Standard and impurity stock solution: 5 mg of dabigatran mesylate/impurity in 10 mL of diluent.

SST Solution: 5 mg of dabigatran and 10 µL of each impurity in to a 10 mL of diluent.
SST Criteria: Since impurity-K eluted near to the analyte peak and which is having the retention time impact based on the pH of a buffer, hence resolution between impurity-K and Dabigatran is monitored as system suitability criteria [17-19].

RESULTS AND DISCUSSION:

Method Development and Optimization: Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G, Imp-H, Imp-I, Imp-J, Imp-K, Imp-L Imp-M, and Imp-N are the potential impurities of dabigatran mesylate drug substance. In this development, the most difficult two challenging things are getting resolution between impurity-A and impurity-B as well as getting consistent retention time for impurity-K**.** Because impurity-K is pH-sensitive and it will merge with impurity-J at lower pH i.e., less than 2.9, and it will move towards the analyte peak at higher pH, *i.e.*, more than 3.0. To get consistent resolution between impurity-K and dabigatran, the pH of the buffer was maintained consistently by fixing the volumes of phosphoric acid as 1.0 mL instead of adjusting the pH to 3.0. The pKa of a drug is the hydrogen ion concentration (pH) at which 50 % of the drug exists in its ionized hydrophilic form. Considering the fact that the pKa value of Dabigatran is highly basic, so it was focused to do the method development attempts in acidic mobile phase to get symmetrical peak shape of the analyte. Method development attempts with different selectivity

using acetonitrile, water, Phosphate buffer, phosphoric acid, and TEA as the mobile phase compositions. The column temperature was played an important role in achieving the resolution between impurities A and B. Where the column temperature is higher, then the resolution between the impurity-A and impurity-B was observed adequately. The gradient program played a significant role in the separation of all 14 impurities with each other. The gradient program was optimized as (T/ % B) is 0/35, 5/35, 30/65, 35/90, and 40/90 with post runtime as 10 min Method development trials were captured in **Table 2**. Method development chromatograms were shown in **Fig. 2**.



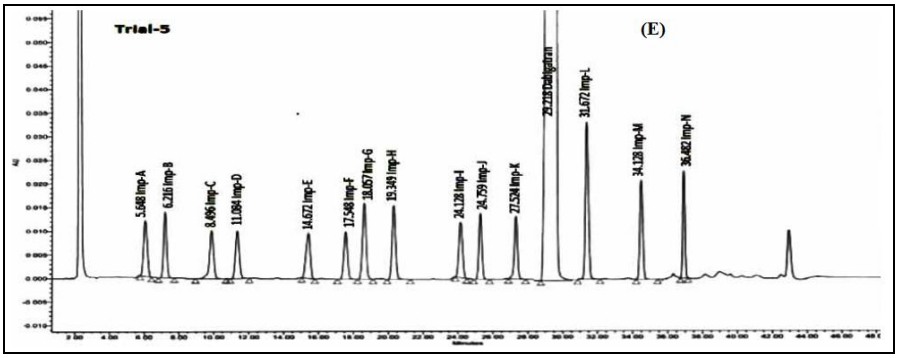


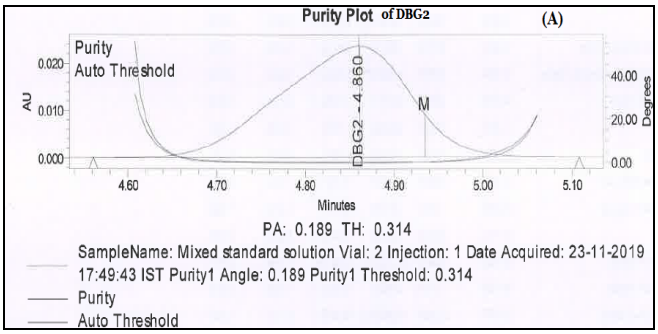
FIG. 2: METHOD DEVELOPMENT CHROMATOGRAMS AT DIFFERENT TRIALS, A)-TRIAL-1, -B)TRIAL-2, -C)TRIAL-3, -D)TRIAL-4, -E)TRIAL-

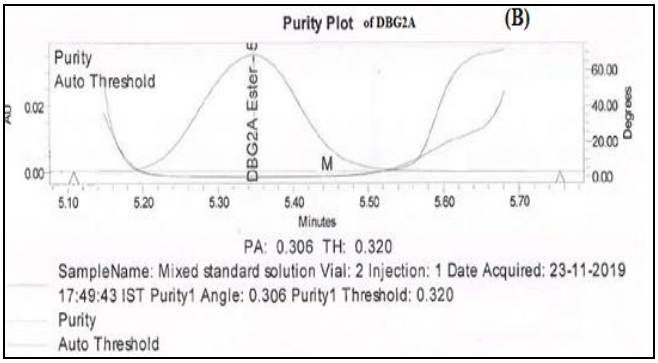
TABLE 2: RESULTS OF METHOD DEVELOPMENT TRIALS

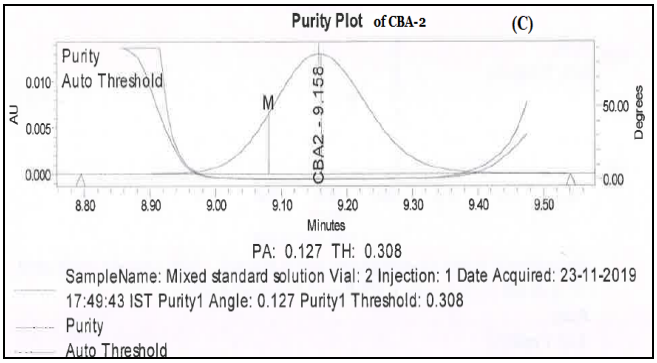
Trial	Column	Dimensions	Mobile phase	Column Temp (°C)	Conclusion
1	Poroshell -SB C8	120×4.6mm ×2.7µ	Ammonium formate buffer /Methanol	25 °C	Imp-A and B are co-eluted and peak shapes are not good
2	Poroshell -EC C18	120×4.6mm ×2.7µ	Phosphate buffer with pH 3.5/Acetonitrile	25 °C	Imp-A and B are coeluted and Imp-K merged with analyte
3	Poroshell -EC C18	120×4.6mm ×2.7µ	Phosphate buffer with pH 2.5/Acetonitrile	25 °C	Imp-K merged with imp-J
4	Poroshell -EC C18	120×4.6mm ×2.7µ	Phosphate buffer with pH 3.0/Acetonitrile	30 °C	Imp-K coeluted with imp-J
5	Poroshell -EC C18	120X4.6mm ×2.7µ	Phosphate Buffer with H3Po4 and TEA addition/Acetonitrile	40°C	Adequate separation between imp-A & Imp-B and Imp-J and Imp-K

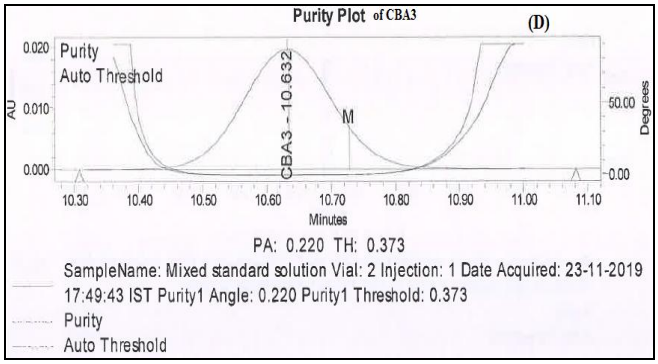
Validation: Analytical method validation for the determination of the related substance of dabigatran drug substance by using HPLC against the ICH Q2 (R1) guideline [20-21].

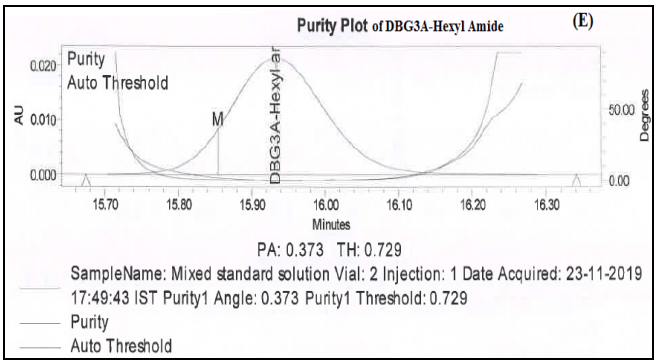
Specificity: Specificity is the ability to assess the analyte unequivocally in the presence of its potential impurities, which may be expected to be present like the other impurities, degradants, matrix, *etc*. The specificity of the developed LC method for Dabigatran was established in the presence of its potential impurities, namely Imp-A, Imp-B, Imp-C, Imp-D, Imp-F, Imp-G, Imp-H, Imp-I, Imp-J, Imp-K, Imp-L, Imp-M, and Imp-N. The ability of the method to separate all of the compounds was assessed by evaluating the peak angle value against the peak threshold of each impurity (Peak angle value is observed lower than the peak threshold for all impurities), which show the Stability-indicating ability and specificity of the proposed rapid LC method. Peak Purity plots of Dabigatran and its impurities were shown in **Fig. 3.** Peak angle and peak threshold values are tabulated in **Table 3.**

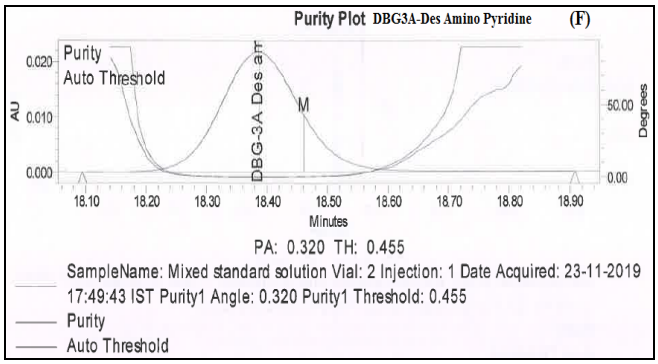


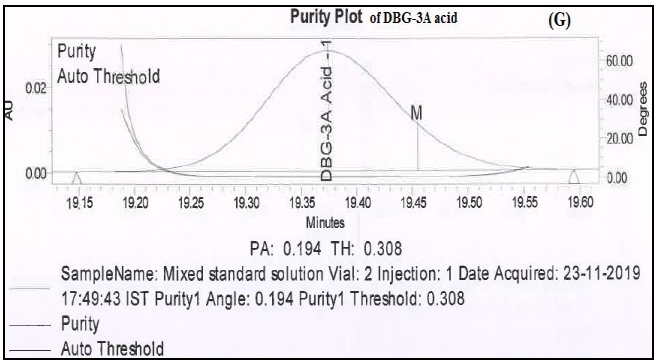


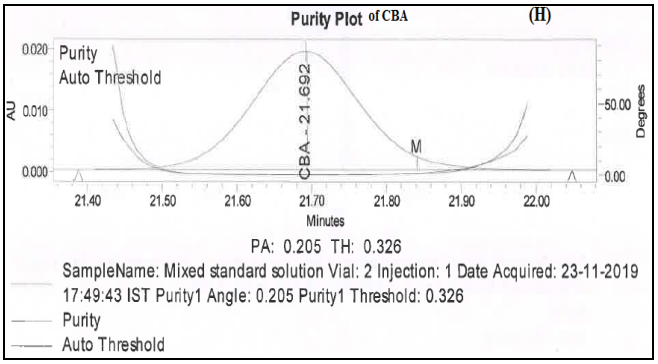


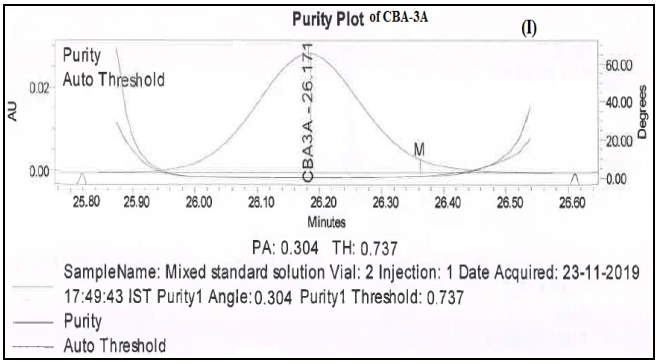

Purity Plot of CBA3
(D)
Purity
Auto Threshold
CBA3 - 10.632
M
AU
Degrees
Minutes
PA: 0.220 TH: 0.373
SampleName: Mixed standard solution Vial: 2 Injection: 1 Date Acquired: 23-11-2019 17:49:43 IST Purity1 Angle: 0.220 Purity1 Threshold: 0.373
Purity
Auto Threshold

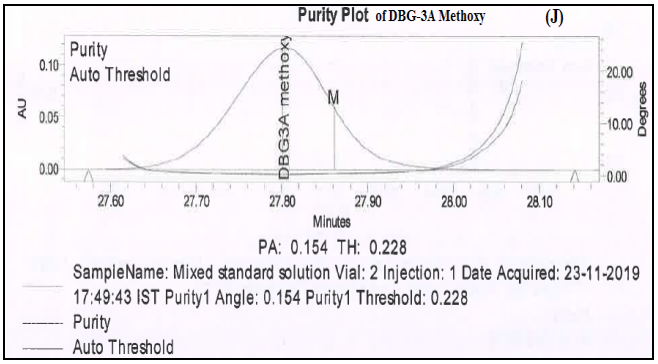

Purity Plot of DBG3A-Hexyl Amide
(E)
Purity
Auto Threshold
DBG3A-Hexyl ar
M
AU
Degrees
Minutes
PA: 0.373 TH: 0.729
SampleName: Mixed standard solution Vial: 2 Injection: 1 Date Acquired: 23-11-2019 17:49:43 IST Purity1 Angle: 0.373 Purity1 Threshold: 0.729
Purity
Auto Threshold

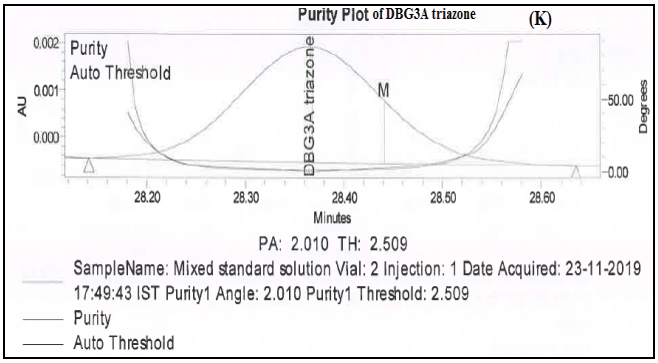

Purity Plot DBG3A-Des Amino Pyridine
(F)
Purity
Auto Threshold
DBG-3A Des am
M
AU
Degrees
Minutes
PA: 0.320 TH: 0.455
SampleName: Mixed standard solution Vial: 2 Injection: 1 Date Acquired: 23-11-2019 17:49:43 IST Purity1 Angle: 0.320 Purity1 Threshold: 0.455
Purity
Auto Threshold

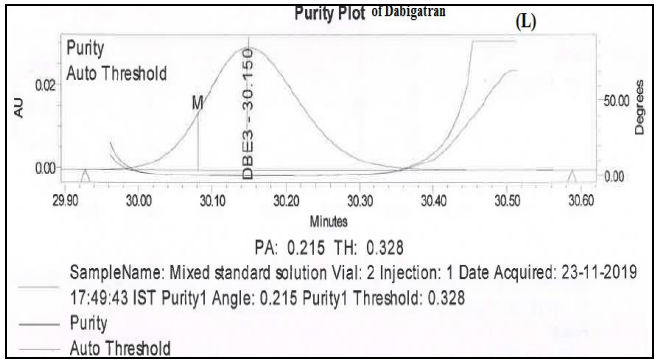

Purity Plot of DBG-3A acid
(G)
Purity
Auto Threshold
DBG-3A Acid -1
M
AU
Degrees
Minutes
PA: 0.194 TH: 0.308
SampleName: Mixed standard solution Vial: 2 Injection: 1 Date Acquired: 23-11-2019 17:49:43 IST Purity1 Angle: 0.194 Purity1 Threshold: 0.308
Purity
Auto Threshold

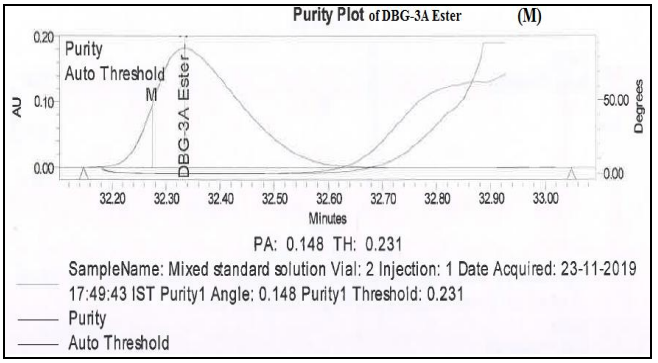

Purity Plot of CBA
(H)
Purity
Auto Threshold
CBA - 21.692
M
AU
Degrees
Minutes
PA: 0.205 TH: 0.326
SampleName: Mixed standard solution Vial: 2 Injection: 1 Date Acquired: 23-11-2019 17:49:43 IST Purity1 Angle: 0.205 Purity1 Threshold: 0.326
Purity
Auto Threshold


Purity Plot of CBA-3A
(I)
Purity
Auto Threshold
CBA3A - 26.171
M
AU
Degrees
Minutes
PA: 0.304 TH: 0.737
SampleName: Mixed standard solution Vial: 2 Injection: 1 Date Acquired: 23-11-2019 17:49:43 IST Purity1 Angle: 0.304 Purity1 Threshold: 0.737
Purity
Auto Threshold


Purity Plot of DBG-3A Methoxy
(J)
Purity
Auto Threshold
DBG3A methoxy
M
AU
Degrees
Minutes
PA: 0.154 TH: 0.228
SampleName: Mixed standard solution Vial: 2 Injection: 1 Date Acquired: 23-11-2019 17:49:43 IST Purity1 Angle: 0.154 Purity1 Threshold: 0.228
Purity
Auto Threshold


Purity Plot of DBG3A triazone
(K)
Purity
Auto Threshold
DBG3A triazone
M
AU
Degrees
Minutes
PA: 2.010 TH: 2.509
SampleName: Mixed standard solution Vial: 2 Injection: 1 Date Acquired: 23-11-2019 17:49:43 IST Purity1 Angle: 2.010 Purity1 Threshold: 2.509
Purity
Auto Threshold


Purity Plot of Dabigatran
(L)
Purity
Auto Threshold
DBE3 - 30.150
M
AU
Degrees
Minutes
PA: 0.215 TH: 0.328
SampleName: Mixed standard solution Vial: 2 Injection: 1 Date Acquired: 23-11-2019 17:49:43 IST Purity1 Angle: 0.215 Purity1 Threshold: 0.328
Purity
Auto Threshold


Purity Plot of DBG-3A Ester
(M)
Purity
Auto Threshold
DBG-3A Ester
M
AU
Degrees
Minutes
PA: 0.148 TH: 0.231
SampleName: Mixed standard solution Vial: 2 Injection: 1 Date Acquired: 23-11-2019 17:49:43 IST Purity1 Angle: 0.148 Purity1 Threshold: 0.231
Purity
Auto Threshold

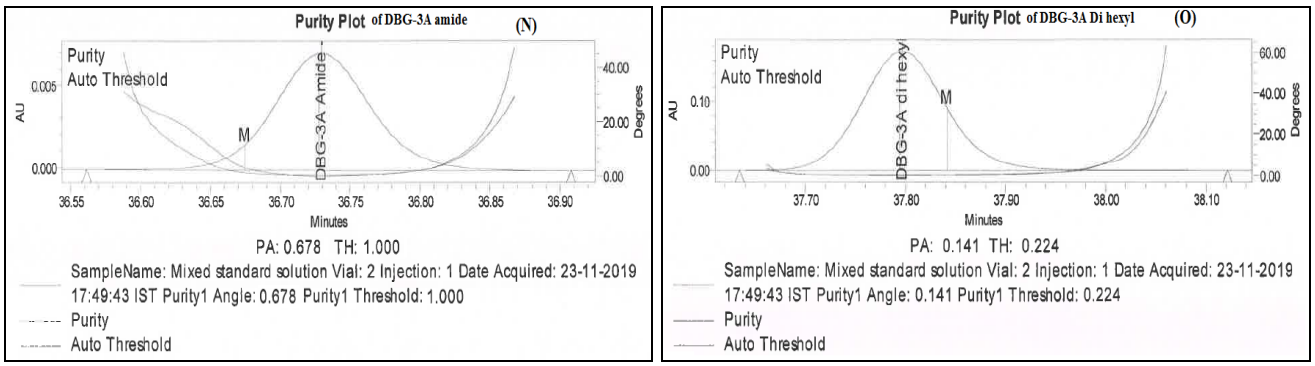


FIG. 3: PURITY PLOTS OF RELATED IMPURITIES A) IMPURITY-A, - B) IMPURITY-B, -C) IMPURITY-C -D) IMPURITY-D, -E) IMPURITY-E, -F) IMPURITY-F, -G) IMPURITY-G, -H) IMPURITY-H, -I) IMPURITY-I, -J) IMPURITY-J, -K) IMPURITY-K, L) DABIGATRAN, -M) IMPURITY-L, -N) IMPURITY-M AND O) IMPURITY-N

TABLE 3: PEAK AND PEAK THRESHOLD VALUES OF DABIGATRAN AND ITS IMPURITIES IN SPECIFICITY

S. no.	Name of the Impurity	Retention time (min)	Relative retention time (RRT)	Peak Angle	Peak Threshold	Peak Purity
1	Impurity-A	4.8	0.17	0.189	0.314	Pass
2	Impurity-B	5.3	0.18	0.306	0.32	Pass
3	Impurity-C	8.3	0.28	0.127	0.308	Pass
4	Impurity-D	9.7	0.33	0.22	0.373	Pass
5	Impurity-E	15.9	0.53	0.373	0.729	Pass
6	Impurity-F	17.9	0.6	0.32	0.455	Pass
7	Impurity-G	18.6	0.62	0.194	0.308	Pass
8	Impurity-H	20.7	0.69	0.205	0.326	Pass
9	Impurity-I	24.6	0.83	0.304	0.737	Pass
10	Impurity-J	25.8	0.87	0.154	0.228	Pass
11	Impurity-K	27.6	0.93	2.01	2.509	Pass
12	Dabigatran	29.8	1	0.215	0.328	Pass
13	Impurity-L	32	1.07	0.148	0.231	Pass
14	Impurity-M	35	1.17	0.678	1.0	Pass
15	Impurity-N	37.7	1.27	0.141	0.224	Pass

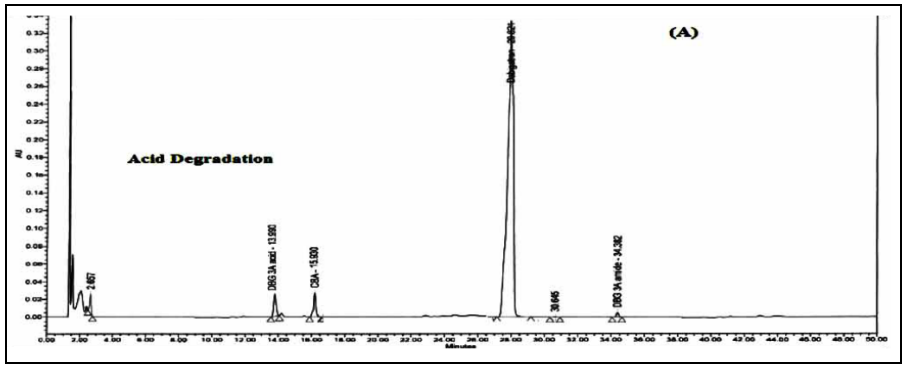
Stress Study: The stress conditions employed for degradation studies as per ICH recommendation include thermal, oxidation, and hydrolysis with acid and base.

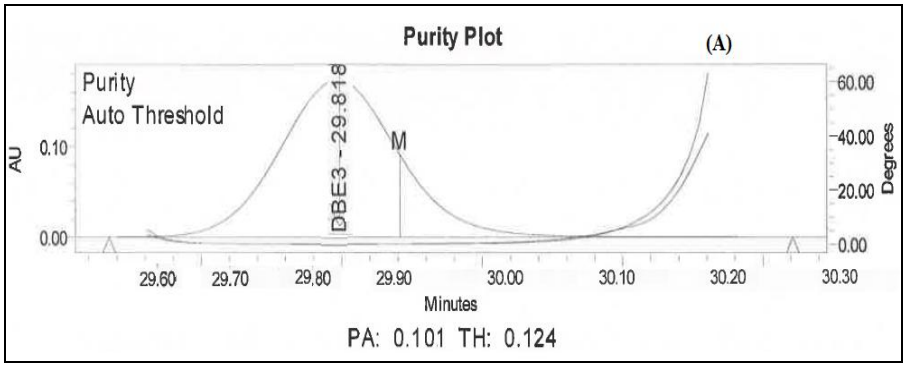
The sample was dissolved in 0.1 N hydrochloric acid and heated under reflux for 30 min for acid degradation study. The sample was dissolved in 1N sodium hydroxide and allowed to stand at room temperature for 30 min for alkali stress conditions. To 1 ml of the sample solution, 1 mL of hydrogen peroxide (1 %) was added.

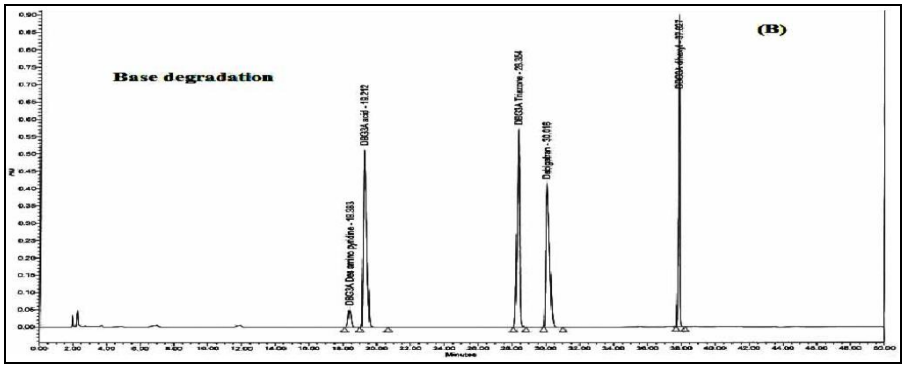
This solution was heated separately for 1 h at 80 °C for temperature stress. The sample was subjected to heat at 105 °C for 72 h. The summary of the forced degradation was captured in **Table 4,** and purity plots were shown in **Fig. 4.**

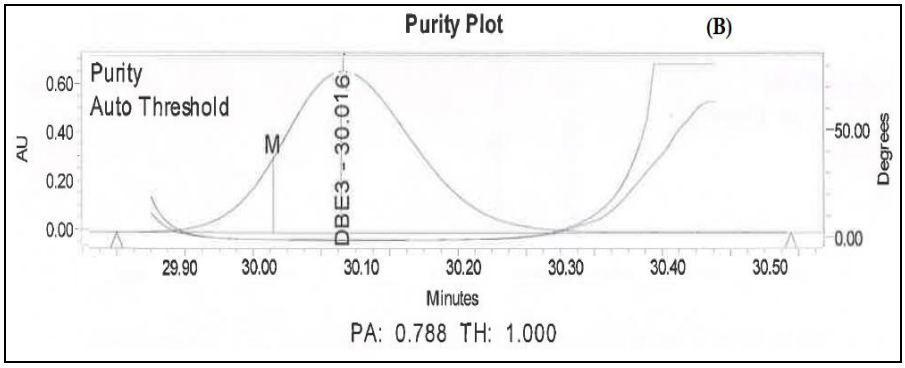
TABLE 4: SUMMARY OF FORCE DEGRADATION RESULTS

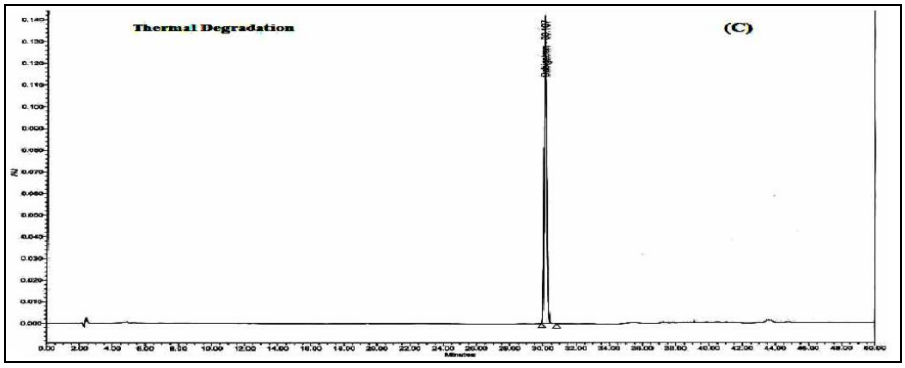
Stress Condition	Time	Purity of Analyte After Degradation	Remarks
Unstressed condition	-	99.7%	-
Acid Hydrolysis (0.1N HCl)	24 h	89.5%	Significant degradation was observed. Impurities G, H and M were formed.
Base Hydrolysis (1N NaOH)	24 h	80.1%	Significant degradation was observed. Impurities F, J, K and N were formed.
Oxidation (1%H_2O_2)	72 h	86.7%	Significant degradation was observed. Impurities C, I, K and M were formed.
Thermal (105 °C)	3 d	99.4%	No degradation products formed


(A)
Acid Degradation


Purity Plot
(A)
Purity
Auto Threshold
DBE3 - 29.818
M
AU
0.10
0.00
60.00
40.00
20.00
0.00
Degrees
29.60
29.70
29.80
29.90
30.00
30.10
30.20
30.30
Minutes
PA: 0.101 TH: 0.124


(B)
Base degradation


Purity Plot
(B)
Purity
Auto Threshold
M
DBE3 - 30.016
AU
0.60
0.40
0.20
0.00
50.00
0.00
Degrees
29.90
30.00
30.10
30.20
30.30
30.40
30.50
Minutes
PA: 0.788 TH: 1.000


Thermal Degradation
(C)

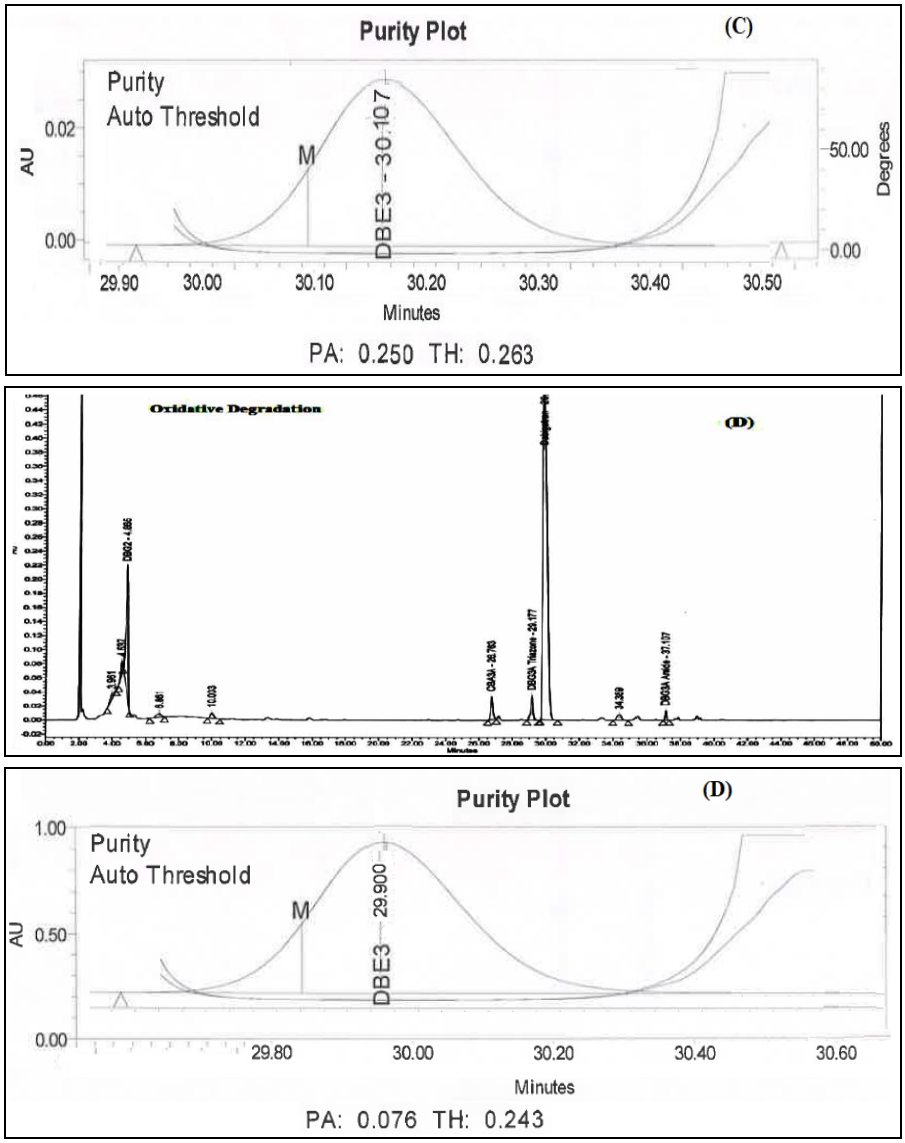


FIG. 4: CHROMATOGRAMS AND PURITY PLOTS OF DABIGATRAN IN PRESENCE OF DEGRADATION PRODUCTS A) ACID DEGRADATION WITH 0.1N HCL B) BASE DEGRADATION WITH 1.0N NaOH C) THERMAL DEGRADATION AT 105°C, D)OXIDATIVE DEGRADATION WITH 1 % H_2O_2

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD and LOQ of dabigatran mesylate and its 14 potential impurities were determined by diluting their stock solutions to known concentration solutions that would yield a signal to noise ratio of 3:1 and 10:1, respectively. Precision was carried out at the LOQ level by preparing six individual preparations of dabigatran with its related impurities at the LOQ level and by calculating the percentage RSD for the areas of dabigatran and its related impurities. Accuracy at the LOQ level was also carried out by preparing three recovery solutions of dabigatran with its related impurities at the LOQ level and by calculating the percentage recovery for areas of all related impurities.

LOD and LOQ results were tabulated in **Table 5**. LOQ recovery results were captured in **Table 6**. Blank, LOD, and LOQ chromatograms are shown in **Fig. 6-8**.

TABLE 5: RESULTS OF LOD AND LOQ

Name of the Impurity	Retention time (~min)	LOD (µg/mL)	S/N Ratio	LOQ (µg/mL)	S/N Ratio	Resolution
Impurity-A	5	62.8	3	190	10.8	-
Impurity-B	5.3	28.5	3.5	86	11.9	2.7
Impurity-C	8.3	38.2	3.7	116	13.5	7.6
Impurity-D	9.7	44.6	3.5	135	16.4	2.8

Impurity-E	15.9	40.3	4.1	122	12	12.1
Impurity-F	17.9	29.6	3.3	90	11.2	4.2
Impurity-G	18.6	35.9	4	109	10.9	2.5
Impurity-H	20.7	43	3.9	130	13.2	3.1
Impurity-I	24.6	42.1	3.4	128	12.8	7.5
Impurity-J	25.8	47.4	3.2	144	14.2	2.3
Impurity-K	27.6	34.7	3.5	105	16.2	3.8
Dabigatran	29.8	45.7	4.2	138	13.9	3.3
Impurity-L	32	32.2	4.2	98	12.4	4.4
Impurity-M	35	47.3	4.5	143	11.6	5.4
Impurity-N	37.7	45.1	3.7	137	10.5	6.5

TABLE 6: VALIDATION RESULTS

Name of The Impurity	%RSD (N=6)	%RSD (N=12)	Correlation Coefficient	%Y-Intercept	LOQ (N=3)	50% (N=3)	100% (N=6)	150% (N=3)
Impurity-A	1.8	2.8	0.9986	-1.9	103	101.7	104.5	103.1
Impurity-B	1.7	1.4	0.9992	0.7	98.8	102.5	102.7	104
Impurity-C	3.6	3.6	0.9989	-1.9	100.6	94.6	104.8	103.5
Impurity-D	3.6	4	0.999	-0.4	95.8	100.5	100.5	97.3
Impurity-E	2.3	2.6	0.9978	-0.9	100.9	102.7	105.1	104.4
Impurity-F	2	1.7	0.9985	0.1	101.8	105.3	102.7	104.1
Impurity-G	1.9	2.4	0.9987	1.2	98.3	104.8	98.1	104.8
Impurity-H	3.9	3.1	0.9987	0.7	100.3	96	101.2	107.1
Impurity-I	4.2	4.6	0.9949	-1.3	103.3	101.2	97.5	102
Impurity-J	2.9	3.5	0.9989	0.5	97.2	104.3	101.3	105.1
Impurity-K	2.2	2.5	0.999	1.5	99.2	98.3	105.3	102.5
Dabigatran	1.6	1.8	0.9995	0.3	NA	NA	NA	NA
Impurity-L	1.2	1.6	0.9988	0.8	103	101.5	100.3	99.4
Impurity-M	2.8	3.7	0.9972	-1	103.7	102.3	101.5	99.3
Impurity-N	3.3	4.3	0.9977	-1.8	99.2	102.1	101.4	102.7

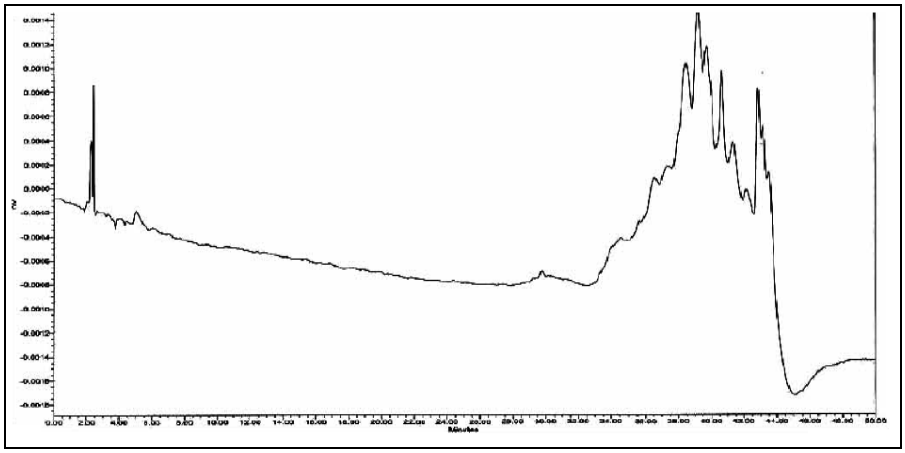


FIG. 6: TYPICAL HPLC CHROMATOGRAM FOR BLANK OF DABIGATRAN

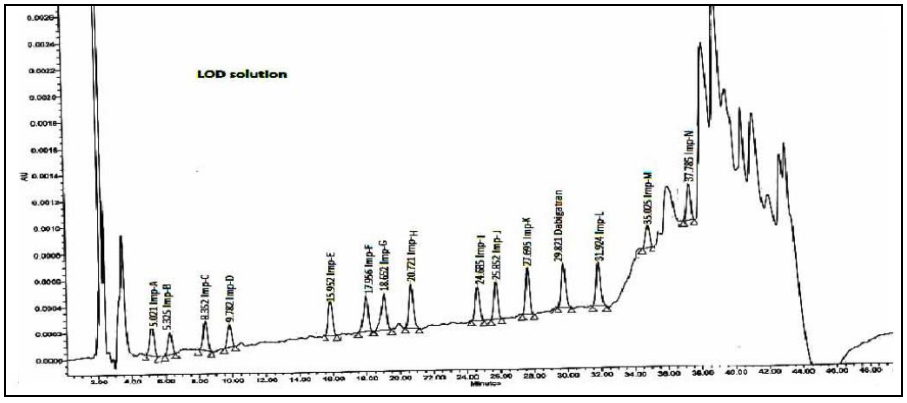


FIG. 7: TYPICAL HPLC CHROMATOGRAM FOR LOD SOLUTION

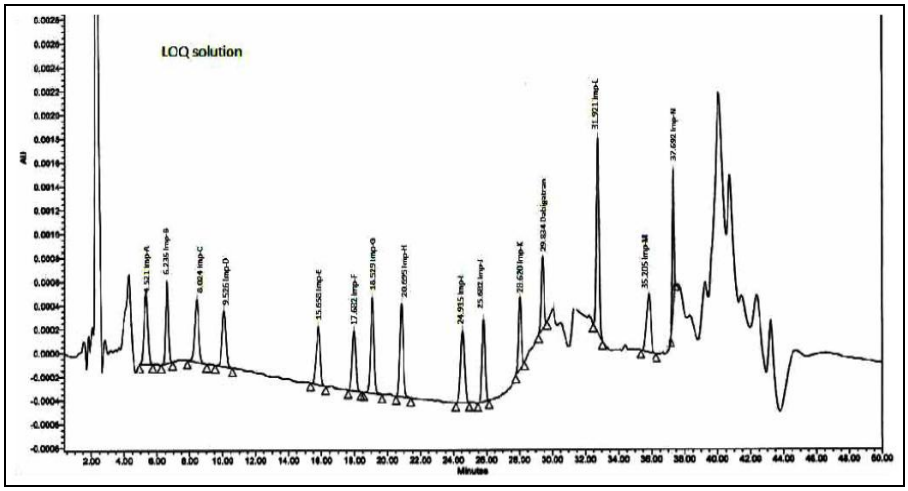
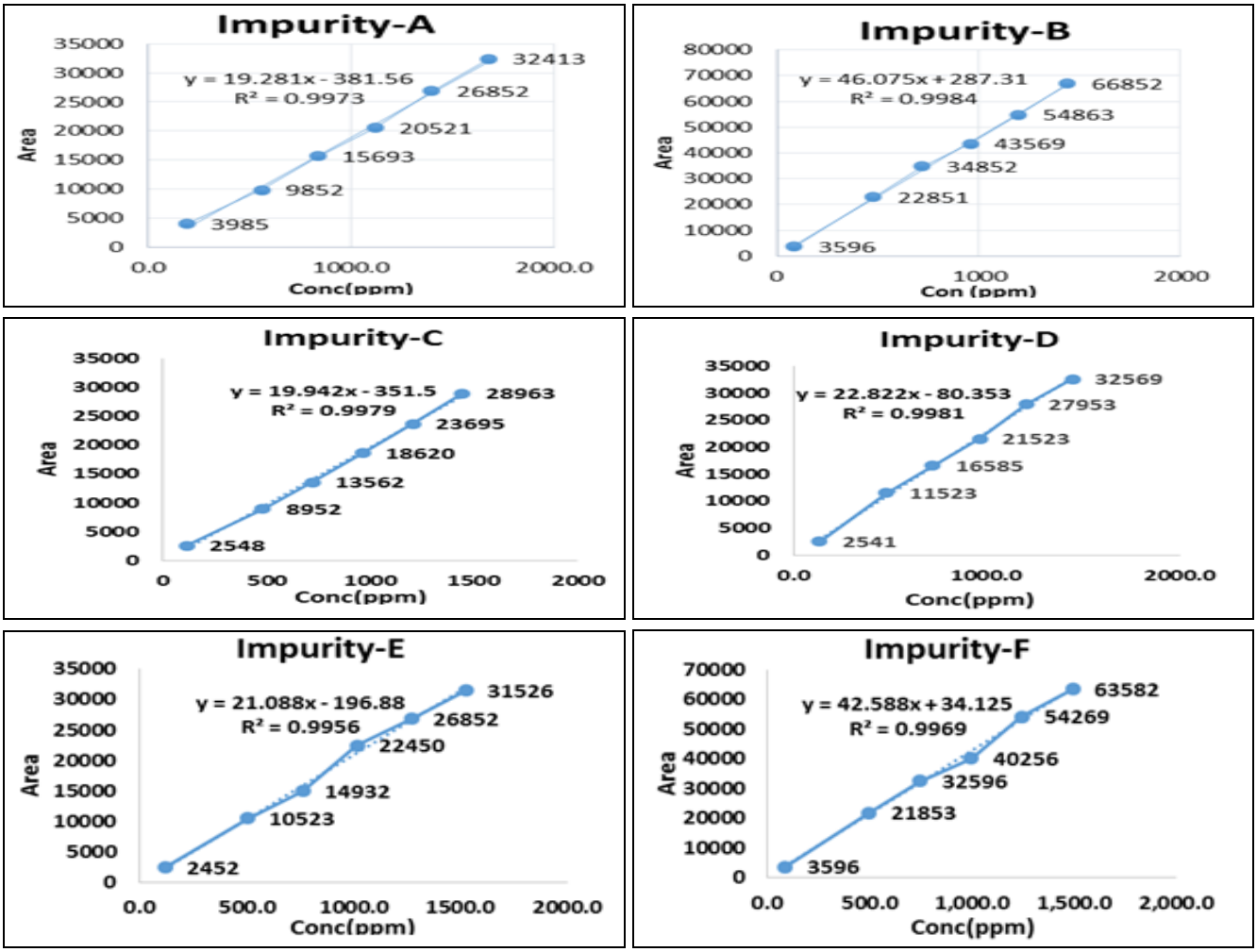


FIG. 8: TYPICAL HPLC CHROMATOGRAM FOR LOQ SOLUTION

Linearity: The linearity at a low level was performed by preparing six different solutions from the LOQ to 0.15% w/w (LOQ, 0.05, 0.075, 0.10, 0.125 and 0.15% w/w) of Imp-A, Imp-B, Imp-C, Imp-D, Imp-F, Imp-G, Imp-H, Imp-I, Imp-J, Imp-K, Imp-L, Imp-M, Imp-N and Dabigatran Mesylate with respect to the target analyte concentration. The peak area versus concentration data was plotted for linear regression analysis. The correlation coefficients of regression slope and intercept and percent y-intercept of the calibration curves were computed. Linearity results are tabulated in **Table 6,** and calibration curves were shown in **Fig. 5**.



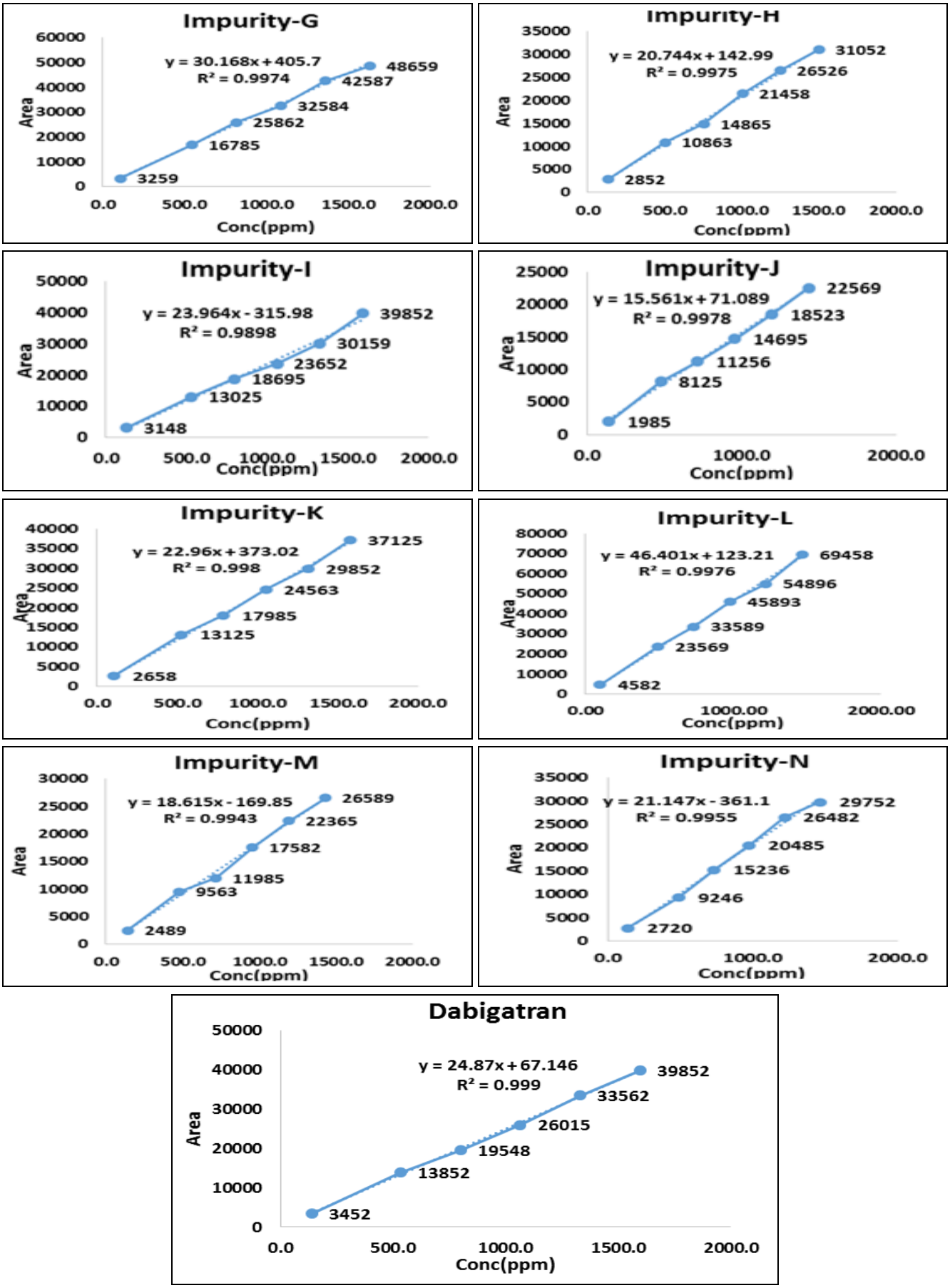


FIG. 9: LINEARITY GRAPHS OF DABIGATRAN MESYLATE AND ITS RELATED IMPURITIES

Precision: The content of each impurity was determined for each of the preparations and the method precision was evaluated by calculating the % RSD of each of the impurity's content in six preparations.

Experiments with a different analyst, column, and instrument in the same laboratory were performed in order to ascertain the intermediate precision or ruggedness of the developed method. Precision results were captured in **Table 6.**

Accuracy: Accuracy was carried out in triplicate at 0.05, 0.10, and 0.15% w/w of the target analyte concentration. The percentage recoveries for each of the 14 impurities were calculated by considering the amount of impurity spiked, amount of impurity available in an un-spiked sample, and amount of impurity recovered. Accuracy results were captured in **Table 6.**

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. To determine the robustness of the developed LC method, deliberate changes were made from original experimental conditions such as flow rate changed to 0.6 and 0.8 mL/min instead of 0.7 nL/min.

The effect of wavelength was studied at 225 nm and 235 nm, instead of at 230 nm. The column temperature was studied at 35 °C and 45 °C instead of 40 °C. The effect of ratio of organic modifier was studied with the variation of ± 10.0% (As such). For all changed conditions, *i.e.*, flow rate, wavelength, temperature, and organic modifier, the system suitability results were recorded. System suitability results are tabulated in **Table 7**.

TABLE 7: SYSTEM SUITABILITY RESULTS IN ROBUSTNESS

Parameter		Resolution Between Dabigatran and Impurity-K
System Suitability		3.2
Robustness		
Flow Variation (0.6 mL/min)		3.8
Flow Variation (0.8 mL/min)		2.9
Column Temperature Variation (35º C)		3.1
Column Temperature Variation (45º C)		3.3
Wavelength at 225		3.1
Wavelength at 235		3.2
MP-A(Buffer: ACN) V/V	95.5::4.5	3.7
	94.5::5.5	3.0
MP-B(Buffer: ACN) V/V	64::36	4.1
	56::44	2.6

Solution Stability and Mobile Phase Stability: Solution stability of the method was evaluated by injection the spiked Dabigatran mesylate standard with known impurities (specification level) at different time intervals. Solution stability was evaluated at 12 h, 24 h, and 48 h and compared with initial results. These solution stability data is indicating that the solution stable up to 48 h, and those results are tabulated in **Table 8**.

The mobile phase stability was carried out by evaluating the analyte and content of all related impurities in the dabigatran mesylate sample solution, which was spiked with known impurities at the specification level (*i.e.*, 0.1 %); the spiked solution was prepared freshly at each 12 h interval up to 48 h and injected, while the same mobile phase was used during the study period. Mobile phase stability results are tabulated in **Table 9.**

TABLE 8: IMPURITY CONTENT AT DIFFERENT TIME INTERVALS IN SOLUTION STABILITY

S. no.	Name of the Impurity	Solution Stability at Different Time Intervals			
		Initial	12hr	24hr	48hr
1	Impurity-A	0.099	0.102	0.098	0.098
2	Impurity-B	0.099	0.099	0.098	0.098
3	Impurity-C	0.1	0.103	0.101	0.101
4	Impurity-D	0.112	0.104	0.111	0.111
5	Impurity-E	0.098	0.098	0.099	0.099
6	Impurity-F	0.104	0.095	0.106	0.106

7	Impurity-G	0.095	0.096	0.092	0.092
8	Impurity-H	0.098	0.097	0.097	0.097
9	Impurity-I	0.092	0.089	0.089	0.089
10	Impurity-J	0.096	0.096	0.096	0.096
11	Impurity-K	0.099	0.097	0.097	0.097
12	Impurity-L	0.098	0.098	0.098	0.098
13	Impurity-M	0.096	0.098	0.098	0.098
14	Impurity-N	0.093	0.101	0.101	0.101

TABLE 9: % VARIATION IN THE IMPURITY CONTENT AT DIFFERENT TIME INTERVALS

S. no.	Name of the Impurity	Mobile phase stability at different time intervals				
		Initial	Day-1	% Variation (1st day)	Day-2	%Variation (2nd day)
1	Impurity-A	0.099	0.098	1	0.101	2
2	Impurity-B	0.099	0.101	-2	0.104	5.1
3	Impurity-C	0.1	0.099	1	0.095	-5
4	Impurity-D	0.112	0.11	1.8	0.109	-2.7
5	Impurity-E	0.098	0.095	3.1	0.093	-5.1
6	Impurity-F	0.104	0.108	-3.8	0.105	1
7	Impurity-G	0.095	0.098	-3.2	0.094	-1.1
8	Impurity-H	0.098	0.095	3.1	0.096	-2
9	Impurity-I	0.092	0.098	-6.5	0.095	3.3
10	Impurity-J	0.096	0.094	2.1	0.1	4.2
11	Impurity-K	0.099	0.095	4	0.096	-3
12	Impurity-L	0.098	0.093	5.1	0.093	-5.1
13	Impurity-M	0.096	0.101	-5.2	0.093	-3.1
14	Impurity-N	0.093	0.098	-5.4	0.09	-3.2

CONCLUSION: A RP-HPLC method for determining the potential impurities which are formed as stage-wise by-products and raw materials during the synthesis of dabigatran mesylate has been successfully developed. This method is having a lot of advantages owing to shorter run time, and it is the single method that can be able to separate fourteen impurities of dabigatran at different stages. This method has also been validated as per ICH guidelines. The method is found to be specific, precise, linear, and accurate in the range of its intended application. Hence, it is suitable for use in routine analysis in any quality control laboratory.

ACKNOWLEDGEMENT: The authors wish to thank the management of Dr. Reddy's Laboratories Ltd. for permitting this work to be published. The cooperation extended by all the colleagues of the Analytical R&D and Process R&D division is gratefully acknowledged.

CONFLICTS OF INTERESTS: The authors have declared no conflicts of interest.

REFERENCES:

1. Paul C: Brown center for drug evaluation and research. Pharmacology Review(s) 2010; 1: 318.
2. Van Ryn J, Hauel N, Waldmann L and Wienen W: Dabigatran inhibits both clot-bound and fluid phase Thrombin *in-vitro* effects compared to Heparin and Hirudin. In ASH Annual Meeting Abstracts 2007; 110: 3998.
3. Laizure SC, Parker RB, Herring VL and Hu ZY: Identification of carboxyl esterase-dependent dabigatran hydrolysis. Drug Metabolism and Disposition 2014; 42: 201-06.
4. Stangier J, Rathgen K, Gansser D and Roth W: The pharmacokinetics, pharmacodynamics and tolerability of dabigatran etexilate, a new oral direct thrombin inhibitor, in healthy male subjects. British Journal of Clinical Pharmacology 2007; 64: 292-03.
5. Khan NA, Ahir KB, Shukla NB and Mishra PJ: A validated stability-indicating reversed phase high performance liquid chromatographic method for the determination of Dabigatran Etexilatemesylate. Invent Rapid Pharma Analysis and Quality Assurance 2014.
6. Sandeep B, Haque MA, Reddy PR and Bakshi PR: Method development and validation for the determination of related compounds in dabigatran etexilate mesylate. International Journal of Medical Nano Research 2014; 1(3): 186-93.
7. Li J, Fang J, Zhong F, Li W, Tang Y, Xu Y, Mao S and Fan G: Development and validation of a liquid chromatography/tandem mass spectrometry assay for the simultaneous deter-mination of dabigatran etexilate, intermediate metabolite and dabigatran in 50 lL rat plasma and its application to pharmacokinetic study. Journal of chromatography B 2014; 973: 110-19.
8. Dare M, Jain R, Pandey A: Method validation for stability indicating method of related substance in active pharmaceutical ingredients dabigatran etexilate mesylate by reverse phase chromatography. J of Chromatography and Separation Techniques 2015; 6(2): 2-10.

9. Bernardi RM, Froehlich PE and Bergold AM: Development and validation of a stability-indicating liquid chromatography method for the determination of dabigatran etexilate in capsules. Journal of AOAC International 2013; 96(1): 37- 41.
10. Sreenivas N, Raghu BK, Douglas SP and Ray UK: Validation of stability-indicating reverse phase HPLC method for the determination of related substances in dabigatran etexilate mesylate drug substance. The pharma letter 2015; 7: 272-9.
11. Nouman EG, Al-Ghobashy MA and Lotfy HM: Development and validation of LC–MSMS assay for the determination of the product dabigatran etexilate and its active metabolites in humanplasma. Journal of Chromatography B 2015; 989: 37-45.
12. Reddy MBS and Rao NVB: A stability indicating RP-HPLC method for estimation of dabigatran in pure and pharmaceutical dosage forms. South Pacif. Journal of Pharmaceutical and Bio Sciences 2014; 2: 080-92.
13. Damle MC and Bagwe RA: Development and validation of stability-indicating RP-HPLC method for estimation of dapigatran etexilate. Journal of Advanced Scientific Research 2014; 5(3): 39-44.
14. Delavennea X, Moracchinia J, Laporteb S, Mismettib P and Basset T: UPLC MS/MS assay for routine quantification of dabigatran - A direct thrombin inhibitor - In human plasma. Journal of Pharmaceutical and Biomedical Analysis 2012; 58: 152-56.
15. Hussain SS, Bhavani G and Kumar AA: UV Spectrophotometric assay method development and validation of dabigatran etexilate in capsules. International J of Pharm and Pharmaceuti Sciences 2015; 7(8): 286-89.
16. Roy S, Patel AB, Ghelani H and Parmar SJ: Development and validation of spectroscopic method for estimation of dabigatran etexilate mesylate in capsule dosage form. International Journal of Pharmacy and Integrated Life Sciences 2014; 2(10): 61-71.
17. Shelke PG and Chandewar AV: Validated stability-indicating high performance liquid chromatographic assay method for the determination of dabigatran etexilate mesylate. J of Pharm Bio and Che Sc 2014; 5(2): 1637-44.
18. Bakshi M and Singh: Development of stability- indicating assay methods-Critical review. Journal of pharmaceutical and biomedical analysis 2020; 28(6): 1011-40.
19. Rao RN and Nagaraju V: An overview of the recent trends in development of HPLC methods for determination of impurities in drugs. Journal of Pharmaceutical and Biomedical Analysis 2003; 33: 335-77.
20. Q2 (R2)/Q14 EWG, 2018, Analytical procedure development and revision of Q2 (R1) analytical validation.
21. ICH, Q7, 2000, Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients.

How to cite this article:
Jhansi TN, Nagaraju R, Kumar DJD and Rao GN: Novel method for separation and quantification of potential impurities by RP-HPLC from KSM stage to API stage of dabigatran mesylate. Int J Pharm Sci & Res 2021; 12(3): 1762-79. doi: 10.13040/IJPSR.0975-8232.12(3).1762-79.