



Method Validation for Stability Indicating Method of Related Substance in Active Pharmaceutical Ingredients Dabigatran Etxilate Mesylate by Reverse Phase Chromatography

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Abstract

To develop the stability indicating, highly accurate precise and linear method for related substance of the Dabigatran through the reverse phase high performance liquid chromatography and it is validated as per the current ICH guideline. The optimize method uses a reverse phase column, Poroshell 120 EC -18 (150 mm × 4.6 mm, 2.7μ), Mobile Phase of hexane-1 Sulfonic acid sodium salt monohydrate (6.5 ± 0.05) and Methanol through gradient flow rate of 0.6ml/min. Keeping the column at temperature 30°C and using the sample amount 10 μL at 5°C and detected all the impurities at 230 nm by the UV detector. In the developed method, elution of Dabigatran was at 26.9 min and all the eluted Impurities were well separated and met the system suitability criteria. The precision is exemplified by relative standard deviation of 0.40%. Method percentage mean recoveries of all the impurities are within the range (90.0% to 115.0%) as per the protocol. Resolution results show that the method is robust. Linearity coefficient for all Impurities is more than 0.999. The LOD obtained is 0.01% and LOQ is 0.03%.

A new highly accurate, precise and stability indicating method was developed for related substance of Dabigatran with all the impurities well separate from the degradation product of Dabigatran. It is ready to use for the routine analysis of related substance of Dabigatran in Pharmaceutical industry.

Keywords: Dabigatran etexilate mesylate; HPLC; Method development; Method validations; ICH

Abbreviations: DBA: Dabigatran Etxilate Mesylate; ICH: International committee of Harmonization; HPLC: High Performance Liquid Chromatography; Impurity-A: 3-(2-((4-Amidinophenylamino)methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamido)propanoic acid; DAB-2B: Ethyl 3-(2-((4-amidinophenylamino)methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamido)propanoate; Impurity-C: Ethyl 3-[[[2-[[[4-[[[(ethylloxy)carbonyl]amino]iminomethyl]phenyl]amino]methyl]-1-methyl-1H-benzimidazol-5-yl]carbonyl](pyridin-2-yl)amino] propanoate; Impurity-D: 3-(2-(((4-(N-((hexyloxy)carbonyl)carbamidoyl)phenyl)amino)methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamido)propanoic acid; Impurity-E: Hexyl ((4-(((5-((3-amino-3-oxopropyl)(pyridin-2-yl)carbamoyl)-1-methyl-1H-benzo[d]idazol-2-yl)methyl)amino)phenyl)(imino)methyl)carbamate; Impurity-G: Ethyl 2-(((4-(N-((hexyloxy)carbonyl)carbamidoyl)phenyl)amino)methyl)-1-methyl-1H-benzo[d]imidazole-5-carboxylate; Impurity-H: 2-(((4-(N-((hexyloxy)carbonyl)carbamidoyl)phenyl)amino)methyl)-1-methyl-1H-benzo[d]imidazole-5-carboxylic acid; Impurity-J: Ethyl 3-(2-(((4-((hexyloxy)carbonyl)carbamoyl)phenyl)amino)methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamido)propanoate; Impurity-K: Ethyl 3-[[[2-[[[4-[[[(pentyloxy)carbonyl]amino]iminomethyl]phenyl]amino]methyl]-1-methyl-1H-benzimidazol-5-yl]carbonyl](pyridin-2-yl)amino]propanoate

Introduction

Dabigatran is a newly available oral direct thrombin inhibitor approved for anticoagulation therapy to prevent strokes in patients with non-valvular arterial fibrillation. It is being studied for various clinical indications and in some cases it offers an alternative to Warfarin as the preferred orally administered anticoagulant ("blood thinner") because it does not require frequent blood tests

for international normalized ratio (INR) monitoring while offering similar results in terms of efficacy. Dabigatran Etxilate chemically Ethyl 3-(1-{2-[[4[Amino({(Hexyloxy)Carbonyl] Imino)}Methyl]Phenyl]Amino)Methyl]-1-Methyl-1H-1,3-Benzodiazol-5-Yl}-N-(Pyridin-2 Yl)Formamido)Propanoate (Figure 1).

Dabigatran (then compound BIBR 953) was discovered from a panel of chemicals with similar structure to benzamide-based thrombin inhibitor α-NAPAP (N-alpha-(2-naphthylsulfonyl)glycyl)-4-amidinophenylalanine piperidine), since 1980s this panel of chemicals had been known as powerful inhibitors of various serine proteases, specifically of thrombin, but also of trypsin [1-3].

The most commonly reported side effect of dabigatran is GI upset. When compared to people anticoagulated with warfarin, patients taking dabigatran had fewer life-threatening bleeds, fewer minor and major bleeds, including intracranial bleeds, but the rate of GI bleeding was significantly higher. Dabigatran capsules contain tartaric acid, which lowers the gastric pH and is required for adequate absorption. The lower pH has previously been associated with dyspepsia; some hypothesize that this plays a role in the increased risk of gastrointestinal

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bleeding [4-10].

Dabigatran has about nine reported impurities chemicals. The chemical structure and the origin of impurities reported under Table 1 [11-13]. Any impurity is defined as a substance coexisting with the original drug, such as starting material or intermediates or that is formed, due to any side reactions. Analysis of impurities in drug substance plays a key role in the novel journey of any drug substance in the pharmaceutical industry. Several analytical method have been reported in literature for the Assay determination of Dabigatran through different analytical methods but no literature reveals the accurate and precise method for the determination of related substances by the RP-HPLC [14-16].

Reverse Phase liquid chromatography has been proven as a versatile, sensitive, reproducible and highly precise method for its ability to separate the complex mixture of Drug Substances with impurities and its easy handling [17,18]. All these advantages of RP-HPLC make this the first choice of modern chemists. So in this scenario a reproducible and accurate method of analysis with proper documented validation give huge support to Pharmaceutical industry.

Material and Methods

A Sample of Dabigatran etexilate mesylate is provided by Micro labs Bangalore with purity of more than 99.0%. All the used Impurities are as per the reference standard. All the solvent and reagent those are used during the analysis are highly pure and HPLC graded. Instrument HPLC with UV/PDA Detector Shimadzu LC-2010CHT, HPLC with UV Detector Waters Alliance Analytical Balance, Micro Balance, PH meter were used during the analysis. All instruments were calibrated prior to Method development and validation.

Buffer preparation

1.88 g hexane-1 sulfonic acid sodium salt monohydrate was dissolved in 1000 mL of water. Add 1 mL of triethylamine to it and adjust the pH to 6.5 ± 0.05 with diluted orthophosphoric acid. Filter through 0.45 μ filter paper.

Time	0.01	15	20	35	45	46	55
% of Mobile phase A	60	30	20	15	15	60	60
% of Mobile phase B	40	70	80	85	85	40	40

Mobile Phase preparation

Mobile phase A prepared as degas Mixture of 20% of methanol and 80% Buffer and Mobile phase B prepared as a degas Mixture 80% methanol and 20% Buffer and both mobile phases use as per the below gradient for obtaining better separation. Poroshell 120 EC -18 (150 mm \times 4.6 mm, 2.7 μ) column is used with flow rate 0.6 ml/min. Keep the column at 30°C and using the sample amount 10 μ L at 5°C and detected all the impurities at 230 nm by the UV detector on the HPLC.

System suitability solution

Accurately weighed about 1 mg each of DAB reference/working standard and DAB Impurity-J reference standard and transferred into a 100 mL volumetric flask, added about 25 mL of diluent, dissolve the sample and make the volume up to 100 ml with diluent. Shake well to mix.

Sample solution

Accurately weigh about 20 mg of sample and transfer into a 100 mL volumetric flask. Add about 25 mL of diluent to it, dissolve and dilute to volume with diluent. Shake well to mix.

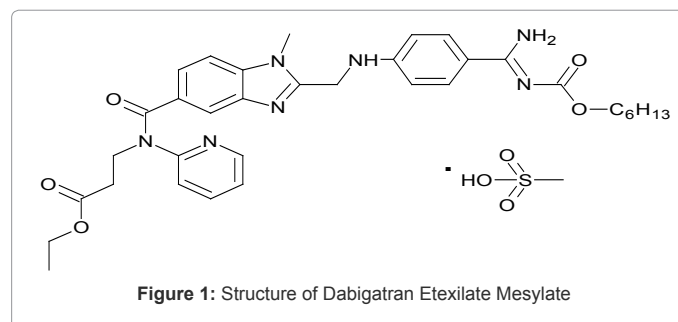


Figure 1: Structure of Dabigatran Etxilate Mesylate

Procedure

The system was Equilibrate until stable baseline was achieved. Inject 2 injection of Blank (Diluent), single injection of system suitability solution and single injection of sample solution. Record the chromatogram and report the result by area normalization method. Eliminate the peaks due to blank. Measure the system suitability criteria. The resolution between DAB and DAB Impurity-J observed in the chromatogram of system suitability solution should be not less than 2.5. Above method was finalized after lots of trial and this method would be validated on different parameters as per the ICH guideline.

Validation procedure and result are described under the result and discussion section.

Method Validation

Solution stability

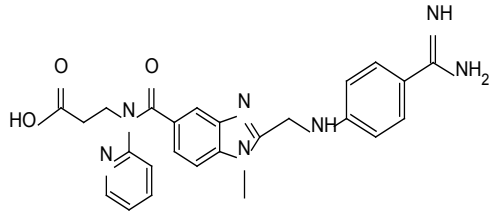
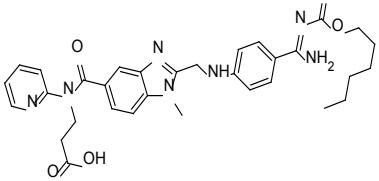
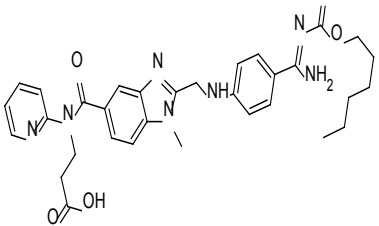
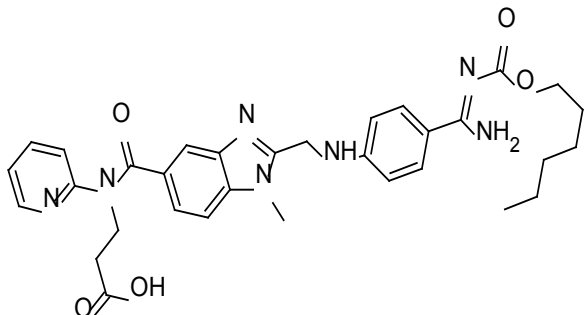
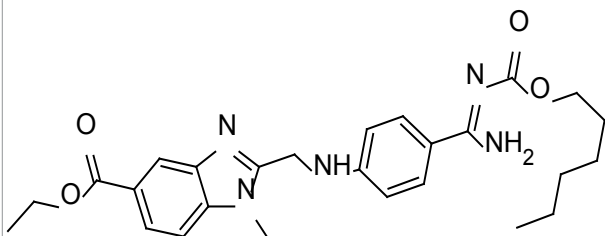
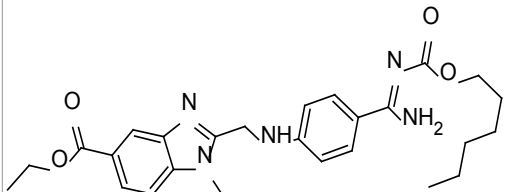
Prepared blank, (as diluents) system suitability solution as per the method and spiked all the impurities which are mentioned in Table 1 as per the specification label. Sample solution was preparing freshly and injected. This study was repeated at the interval of 6 hours till 24 hours to measure the solution stability at 5°C temperature.

Specificity

Specificity is the ability to assess unequivocally of analytic in the presence of components which may be expected to be present. In this study Specificity has been performed as interference study and forced degradation study using HPLC system with PDA detector. For the interference study of DAB drug substance Prepared and injected blank, system suitability solution and spiked all the Impurities as per specification label. Checked the system suitability criteria and recorded the observations.

Force degradation

It is a measurement of specificity and it's also help to proper selection of stability-indicating analytical procedures. A literature reveal the Stress studies should be done in different pH solutions, in the presence of oxygen and light, and at elevated temperatures and humidity levels to determine the stability of the drug substance. These stress studies are conducted on a single batch. This information will in turn help improve the formulation manufacturing process and determine the storage conditions. As no specific set of conditions is applicable to all drug products and drug substances and the regulatory guidance does not specify about the conditions to be used, this study requires the experimenter to use common sense [13]. In our study, stress conditions were performed in presence of Acid, Alkali, Oxygen and light (Photolytic), temperature/humidity and thermal conditions.

S.No	Impurity Code	Structure	Chemical Name	Remarks
1	DAB Impurity -A		3-(2-((4-Amidinophenylamino)methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamido)propanoic acid	Process related , and Metabolite
2	DAB Impurity -B		Ethyl 3-(2-((4-amidinophenylamino)methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamido)propanoate	Process related, Degradation (oxidative) and Metabolite
3	DAB Impurity -C		Ethyl 3-[[[2-[[[4-[[[(ethyloxy)carbonyl]amino]iminomethyl]phenyl]amino]methyl]-1-methyl-1H-benzimidazol-5-yl]carbonyl](pyridin-2-yl)amino]propanoate	Process related
4	DAB Impurity -D		3-(2-(((4-(N-((hexyloxy)carbonyl) carbamimidoyl)phenyl)amino)methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamido)propanoic acid	Process related , degradation (acid,base) and Metabolite
5	DAB Impurity -E		Hexyl ((4-(((5-((3-amino-3oxopropyl)(pyridin-2yl)carbamoyl)-1-methyl-1H-benzo[d]imidazol-2yl)methyl)amino)phenyl)(imino)methyl)carbamate	Process related
6	DAB Impurity -G		Ethyl 2-(((4-(N-((hexyloxy)carbonyl) carbamimidoyl)phenyl)amino)methyl)-1-methyl-1H-benzo[d]imidazole-5-carboxylate	Process related

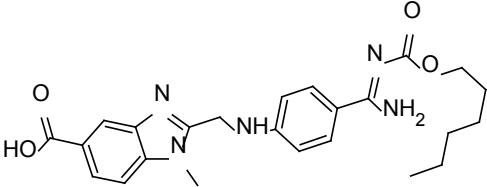
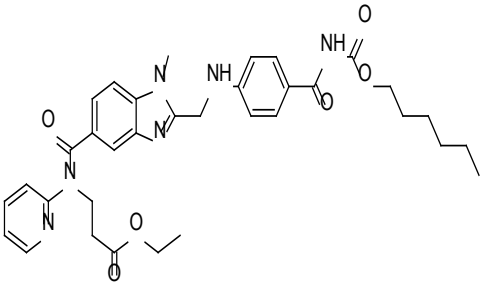
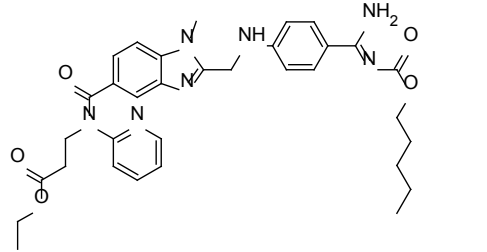
7	DAB Impurity -H		2-(((4-(N-((hexyloxy)carbonyl) carbamimidoyl)phenyl)amino)methyl)-1-methyl-1H-benzo[d]imidazole-5-carboxylic acid	Process related
8	DAB Impurity -J		Ethyl 3-(2-(((4-(N-((hexyloxy)carbonyl) carbamoyl)phenyl)amino)methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamido)propanoate	Process related and Degradation (Temp. & humidity)
9	DAB Impurity -K		Ethyl 3-[[[2-[[[4-[[[[(pentyloxy)carbonyl]amino]iminomethyl]phenyl]amino]methyl]-1-methyl-1H-benzimidazol-5-yl]carbonyl](pyridin-2-yl)amino]propanoate	Process related

Table 1: Origin and pathway of DBA impurities

Limit of detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. The detection limit (DL) may be expressed as [19-21]:

$$DL = 3.3 \sigma$$

S

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways.

Limit of quantization

The quantitation 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 determined by several methods [22,23].

The detection limit (DL) may be expressed as:

$$QL = 10 \sigma$$

S

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways.

In presented study author Determined the limit of quantification (LOQ) of DAB (un-known impurity) by the analysis of samples with known concentrations of analyte by establishing the concentration which will give a concentration of about 3.0 times of LOD with an RSD of 10% for peak area of DAB peak. The % RSD of 6 replicate injection of spiked sample is less than 4%.

Linearity

A linear relationship should be evaluated across the range (see section 3) of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range. As per the ICH guideline Linearity established for all the Impurities and for DAB form different concentration levels that are start form LOQ to 50%, 80%, 100%, 120%, 150% of Impurities as per their shelf specification level [24,25].

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. In the present study author check the accuracy for all the Impurities as well as DAB on different specification level that are LOQ, 50%, 100% and

150%. All the preparation of test solution were prepared in triplicate (n=3).

Precision

It was performed by carrying out the analysis of the six homogenous solutions of same test sample and content of impurities was calculated. The determinations were carried out one after the other under conditions as similar as possible.

Intermediate precision

The intermediate precision of the method was checked by determining precision on a different instrument, analysis being performed in on a different day. The % content of all DAB impurities and total impurities was found to be below quantification level even when it is performed on a different instrument. The method is said to be precise with respect to the criteria of the intermediate precision.

Robustness

According to ICH, 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 use. To establish the robustness of method here, make the changes in HPLC method as per the ICH guidelines. These changes are listed below. Compare the resolution of all Impurities in each condition and data are listed under Table 10

Condition (1): Altered the mobile phase flow by ± 0.1 mL/minute

- Altered the mobile phase flow to 0.5 mL instead of 0.6 mL.
- Altered the mobile phase flow to 0.7 mL instead of 0.6 mL.

Condition (2): Altered the column oven temperature by $\pm 2^\circ\text{C}$

- Altered the column oven temperature to 28°C instead of 30°C .
- Alter the column oven temperature to 32°C instead of 30°C .

Condition (3): Altered the column with different lot number.

Condition (4): Altered the pH of the buffer solution by ± 0.2

- Altered the pH of the buffer solution to 6.30 instead of 6.50.
- Altered the pH of the buffer solution to 6.70 instead of 6.50

Result and Discussion

The present investigation optimum result was obtained under the prescribed chromatographic condition. Retention time of DAB was obtained around at 26.97 min that is well separated from eluting peak of DAB Impurity J. Obtained resolution between DAB and DAB impurity J is 10.02 that are more than required system suitability criteria under the procedure (Figure 2).

The Solution stability results indicate that system suitability solution, sample solution is stable at 5°C up to 18 hours from the time of preparation. All obtained result summarize in Table 2. Specificity result indicate no interference observed at the retention times of known impurities due to blank. Observed purity threshold is more than purity angle for all known impurities and DAB peak in the spiked sample solution All the Result of peak purity shown under (Table 2, Figure 3). So it is concluded the method is specific and selective.

The selection of stress study for DAB is given in (Tables 3 and

3.1). The acceptance criteria is, it should meet the system suitability requirement as per the method as well as percent degradation should be between 5 to 30% and Peak purity of main compound and known impurity peaks should pass. The peak purities of all known impurity peaks and DAB peak in each degradation condition are meeting the criteria. It indicates that there is no co-elution at the retention times of any of the known impurities and DAB peak. Dabigatran Etxilate Mesylate drug substance shows significant degradation in basic, oxidative conditions and slight degradation shown in acid degradation.

In the study of limit of detection injected spiked solution of impurities, we observed the chromatograms and conclude the System suitability criteria of DAB peak is pass. The result of LOD is given in Table 4.

In presented study author Determined the limit of quantification (LOQ) of DAB (un-known impurity) by the analysis of samples with known concentrations of analyte by establishing the concentration which will give a concentration of about 3.0 times of LOD with a RSD of 10% for peak area of DAB peak. The %RSD of 6 replicate injection of spiked sample is less than 4% , all the results of LOD is tabulated in Table 4. The results of LOQ (LOQ for all known impurities and DAB peak is 0.03%) determination indicate that the method is capable of quantitatively determining analytic in a sample with suitable precision and accuracy.

The correlation coefficient obtained was greater than 0.999 for all the impurities and for DAB. This shows that an excellent correlation existed between the peak area and the concentration of impurity A, impurity B, impurity C, impurity D, impurity E, Impurity G, Impurity H, Impurity J, Impurity K and DAB. Calculate the relative response factor from the linearity curve that is shown in Table 5.

Linearity result shows in Table 6 that indicate Correlation co-efficient is not be less than 0.99 for all known impurities and Dabigatran Etxilate Mesylate peak .The % y-intercept as obtained from the linearity data in range of ± 3 for all impurities as well as for the Dabigatran Etxilate Mesylate . So results indicate that the method has ability to generate responses which are directly proportional to the concentration of analytes present in the sample (Figure 4). The Accuracy result is shown in Table 7. All the results indicate that the method has expressed the closeness of agreement between observed value and true value. Hence, Accuracy has been established across the specified range (LOQ to 150%) of analytical method.

The relative standard deviation was calculated during the study of precision and all the obtained observations given under the Table 8 that concludes that method is precise.

Under the intermediate precision calculate the % content of all DAB impurities and total impurities was found to be below quantification level even when it is performed on a different instrument. The method is said to be precise with respect to the criteria of the intermediate precision. Obtained result shown in Table 9.

The results of robustness study indicate that the method is capable of demonstrating its capacity to remain unaffected by small variations in method parameters and provided an indication of its reliability during normal usage. All the result of robustness study summarized under the Table 10.

Conclusion

The analytical method used for determination of related substances in Dabigatran Etxilate Mesylate complies with the acceptance criteria

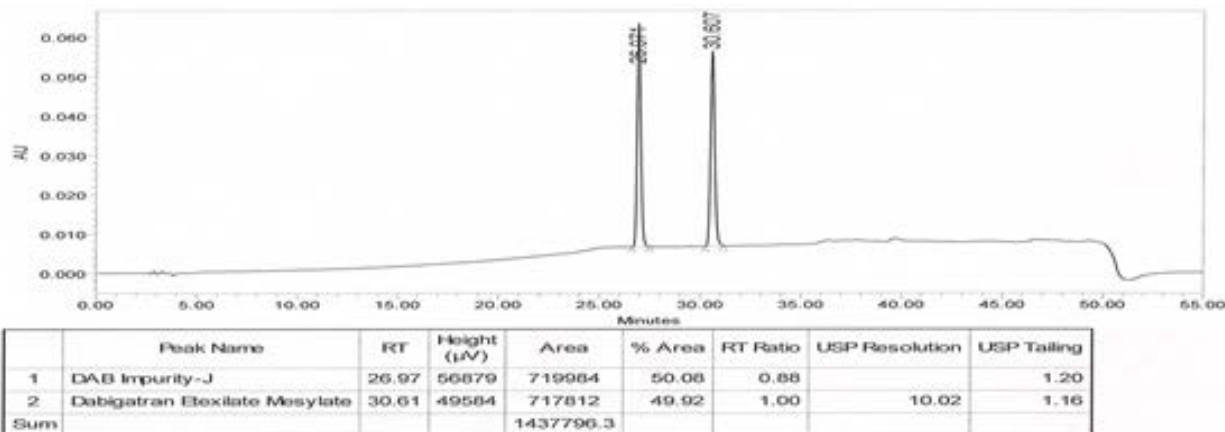
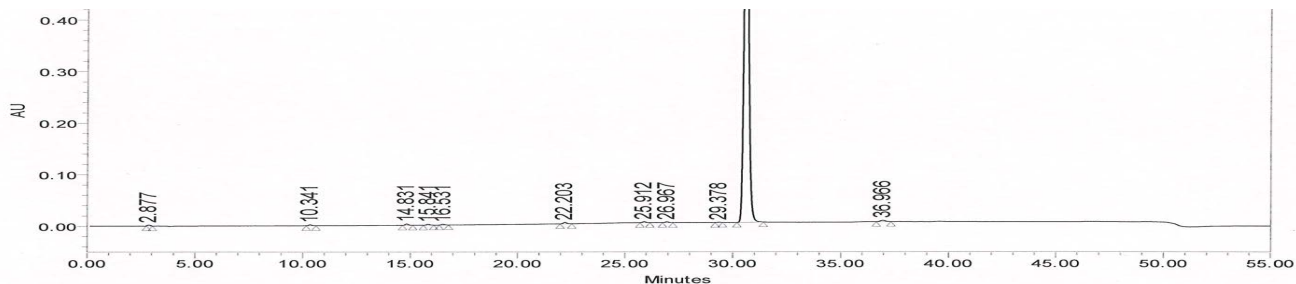


Figure 2: System Suitability Chromatograph



Peak Name	RT	Height (μV)	Area	% Area	RT Ratio	Purity 1 Angle	Purity 1 Threshold
1 DAB Impurity-A	2.88	1740	17650	0.15	0.09	4.660	4.755
2 DAB-2B	10.34	1796	20422	0.17	0.34	2.018	5.362
3 DAB Impurity-C	14.83	2578	28665	0.24	0.48	1.571	4.810
4 DAB Impurity-H	15.84	1757	20170	0.17	0.52	2.547	5.949
5 DAB Impurity-D	16.53	1605	17569	0.15	0.54	2.265	5.669
6 DAB Impurity-E	22.20	2798	32234	0.27	0.73	1.497	4.710
7 DAB Impurity-K	25.91	1880	21522	0.18	0.85	3.516	5.428
8 DAB Impurity-J	26.97	1528	18202	0.15	0.88	2.552	5.745
9 Peak9	29.38	416	4509	0.04	0.96	7.751	12.194
10 Dabigatran Etxilate Mesylate	30.62	807309	11583796	98.09	1.00	0.111	3.008
11 DAB Impurity-G	36.97	2606	44282	0.37	1.21	2.227	5.339
Sum			11809020.0				

Figure 3: Chromatograph shown the Specificity as spiked all the impurities in sample solution

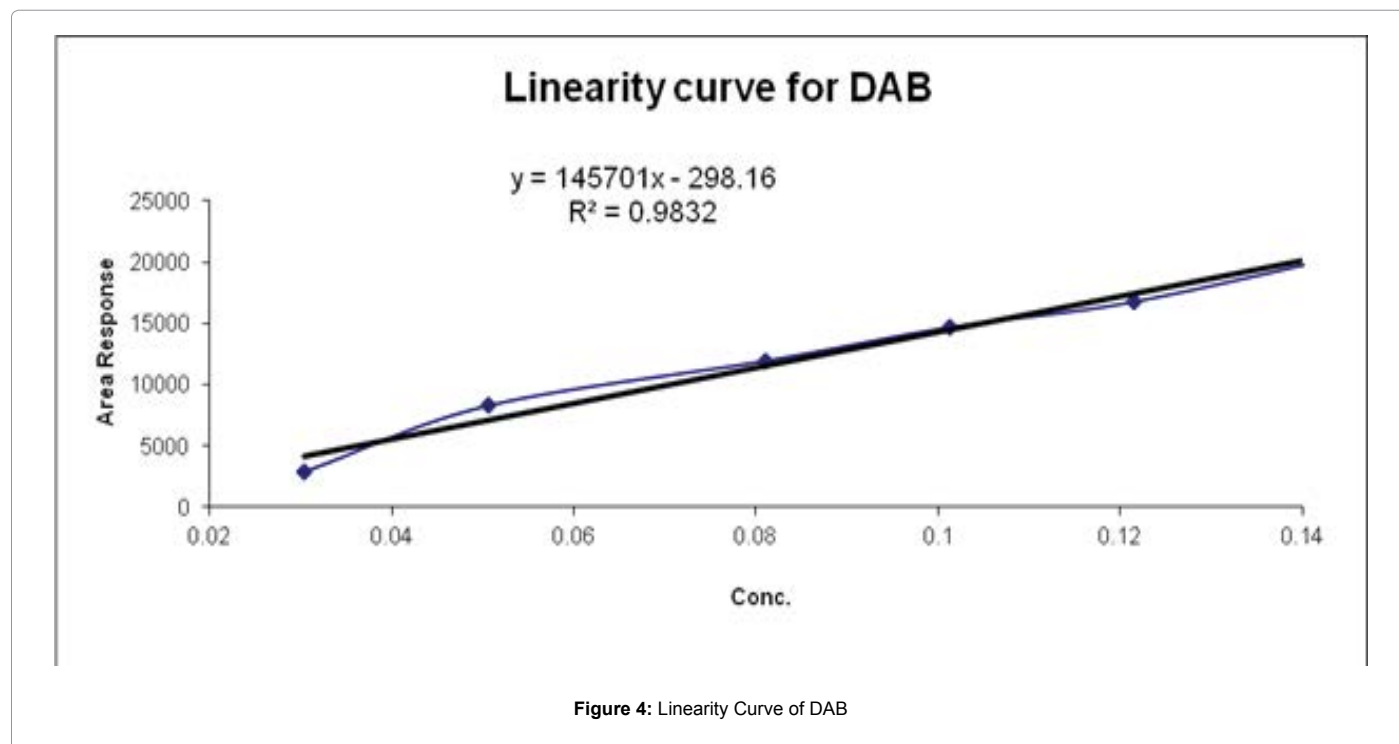


Figure 4: Linearity Curve of DAB

Name of component	Initial	After 6 hours	After 12 hours	After 18 hours	After 24 hours
DAB Impurity-A	-	-0.72	-1.24	-0.68	4.42
DAB-2B	-	0.95	-1.70	-0.45	-0.90
DAB Impurity-C	-	0.00	0.98	-0.24	-1.14
DAB Impurity-H	-	-0.51	-0.53	-1.05	-0.41
DAB Impurity-D	-	0.44	-1.36	0.66	-0.27
DAB Impurity-E	-	-0.91	-0.43	-0.97	-1.30
DAB Impurity-K	-	0.18	-0.60	-0.66	-0.01
DAB Impurity-J	-	-2.34	-7.19	-8.60	-15.89
DAB Impurity-G	-	1.75	1.88	-1.24	-2.61
DAB	-	0.01	0.01	0.03	0.03

Table 2: Summarize the result study of Solution stability of impurities in sample solution.

S. No.	Name of impurity	Conditions for degradation			
		Control sample (%)	Acid degradation (1N HCl / at RT / 1Hour)	Base degradation (1N NaOH / at RT/ 2 hours)	Oxidative degradation (3%v/v H ₂ O ₂ / at RT / 12 Hours)
1	DAB Impurity-A	ND	ND	0.31	
2	DAB-2B	ND	0.01	0.01	1.93
3	DAB Impurity-C	0.08	0.08	0.06	0.08
4	DAB Impurity-H	ND	0.04	0.04	0.25
5	DAB Impurity-D	ND	4.55	9.37	0.04
6	DAB Impurity-E	0.09	0.11	0.09	0.77
7	DAB Impurity-K	0.01	0.01	0.01	0.01
8	DAB Impurity-J	ND	0.03	0.01	0.27
9	DAB Impurity-G	0.04	0.05	0.03	0.71
10	Total unknown impurities	0.08	0.12	0.14	1.30
11	Total impurities	0.30	4.99	10.07	5.36
12	% of Degradation	NA	4.70	9.80	5.07

Table 3: Result of Force Degradation study in Dabigatran etexilate mesylate

S. No.	Name of impurities	Photolytic degradation (%) (1.2Million lux hours & 200 WH/m2)	Temperature/ Humidity degradation (%) (60°C ± 2 and 80%RH ± 5% for 48 hours)	Thermal degradation (%) (105°C±2 for 24 hours)
1	DAB Impurity-A	ND	ND	ND
2	DAB-2B	ND	0.04	0.21
3	DAB Impurity-C	0.09	0.08	0.10
4	DAB Impurity-H	ND	ND	ND
5	DAB Impurity-D	0.02	ND	ND
6	DAB Impurity-E	ND	0.09	0.11
7	DAB Impurity-K	0.01	0.01	ND
8	DAB Impurity-J	0.02	1.32	0.02
9	DAB Impurity-G	0.06	0.04	0.71
10	Total unknown impurities	0.39	0.12	0.10
11	Total impurities	0.59	1.71	1.24
12	% of Degradation	0.29	1.41	0.94

Table 3.1: Result of Force Degradation study in Dabigatran etexilate mesylate

Name of impurity	LOD (%w/w)	Area of injection-1	Area of injection-2	Area of injection-3
DAB Impurity-A	0.01	2069	1930	1906
DAB-2B	0.01	1380	1359	1270
DAB Impurity-C	0.01	1362	1188	1067
DAB Impurity-H	0.01	1531	1460	1513
DAB Impurity-D	0.01	1208	1129	1147
DAB Impurity-E	0.01	1100	1336	1305
DAB Impurity-K	0.01	1190	1124	1169
DAB Impurity-J	0.01	1938	1766	1948
Dabigatran Etxilate Mesylate	0.01	2914	2286	3114
DAB Impurity-G	0.01	1656	1273	1334

Table 4: Result of the study of Limit of detection

Name of impurity	LOQ (%w/w)	Area of inj. -1	Area of inj. -2	Area of inj. -3	Area of inj. -4	Area of inj. -5	Area of inj. -6	% RSD
DAB Impurity-A	0.03	5432	5435	5448	5420	5541	5547	1.05
DAB-2B	0.03	3759	3720	3670	3626	3653	3633	1.43
DAB Impurity-C	0.03	3760	3750	3716	3843	3750	3656	1.64
DAB Impurity-H	0.03	4608	4635	4669	4451	4563	4514	1.77
DAB Impurity-D	0.03	3388	3488	3349	3327	3373	3428	1.71
DAB Impurity-E	0.03	4002	3873	3767	3896	3821	3893	2.04
DAB Impurity-K	0.03	3953	3892	3824	3949	4069	4035	2.28
DAB Impurity-J	0.03	3403	3358	3504	3331	3443	3478	1.99
Dabigatran Etxilate Mesylate	0.03	2910	2883	2805	3031	2907	3032	3.03
DAB Impurity-G	0.03	6703	6792	6804	6789	6989	6899	1.46

Table 5: Result of the study of Limit of Quantization

Name	Limit of detection (% w/w)	Limit of quantitation (%w/w)	Retention time (minutes)	Relative retention time	Relative response factor	Co-relation coefficient (R ²)
DAB Impurity-A	0.01	0.03	2.88	0.09	1.30	1.0000
DAB-2B	0.01	0.03	10.34	0.34	0.91	0.9962
DAB Impurity-C	0.01	0.03	14.83	0.48	0.93	1.0000
DAB Impurity-H	0.01	0.03	15.84	0.52	1.42	1.0000
DAB Impurity-D	0.01	0.03	16.53	0.54	0.93	0.9999
DAB Impurity-E	0.01	0.03	22.20	0.73	0.95	1.0000
DAB Impurity-K	0.01	0.03	25.91	0.85	0.96	0.9979
DAB Impurity-J	0.01	0.03	26.97	0.88	0.86	0.9958
Dabigatran Etxilate Mesylate	0.01	0.03	30.62	1.00	1.0	0.9832
DAB Impurity-G	0.01	0.03	36.97	1.21	1.70	0.9895

Table 6: Result of Linearity and relative response factor for all impurities

Sno.	Name of Impurity/ Analyte	Accuracy @ % LOD Level	Accuracy @ 50% Level	Accuracy @100% Level	Accuracy @150% Level
1.	DAB Impurity-A	109.52	115.23	91.85	115.55
2.	DAB Impurity-B	114.94	110.96	113.56	111.62
3.	DAB Impurity-C	103.57	110.33	111.50	114.55
4.	DAB Impurity-D	109.88	110.78	115.93	110.05
5.	DAB Impurity-E	95.44	113.51	113.15	111.59
6.	DAB Impurity-H	101.45	112.87	113.57	115.88
7.	DAB Impurity-K	113.10	109.52	114.09	116.19
8.	DAB Impurity-J	113.79	115.74	116.43	112.29
9.	DAB Impurity-G	110.34	112.33	115.63	115.75

Table 7: Mean (n=3) Result of Accuracy @ different level with respect to specification level

Sno.	Name of Impurity/ Analyte	% Mean (n=6) of each Impurity	%RSD (n=6) of total Impurity
1.	DAB Impurity-A	0.128	1.618 n=6 Preparation
2.	DAB Impurity-B	0.164	
3.	DAB Impurity-C	0.225	
4.	DAB Impurity-D	0.128	
5.	DAB Impurity-E	0.265	
6.	DAB Impurity-H	0.132	
7.	DAB Impurity-K	0.160	
8.	DAB Impurity-J	0.166	
9.	DAB Impurity-G	0.194	

Table 8: Mean (n=6) Result of precision of six different preparations

Preparation	Total impurities (%)	
	Analyst-1	Analyst-2
Preparation-01	1.625	1.664
Preparation-02	1.611	1.668
Preparation-03	1.614	1.666
Preparation-04	1.612	1.664
Preparation-05	1.622	1.664
Preparation-06	1.625	1.664
Mean	1.618	1.665
% RSD	0.41	0.10
Cumulative mean	1.642	
Cumulative %RSD	0.40	
Acceptance criteria for %RSD	Not more than 10.0%	

Table 9: Result of Intermediate precision

S. No.	Name of component	Resolution					Column change
		Idea condition	Flow decrease	Flow increase	Temp. decrease	Temp. increase	
1	DAB Impurity-A	-	-	-	-	-	-
2	DAB-2B	38.83	36.91	37.87	39.15	37.40	34.00
3	DAB Impurity-C	21.34	20.13	21.80	21.47	21.97	21.54
4	DAB Impurity-H	4.15	4.04	3.31	4.58	3.91	3.83
5	DAB Impurity-D	3.13	2.73	3.41	2.97	3.11	3.21
6	DAB Impurity-E	26.79	24.38	26.67	26.48	25.89	27.29
7	DAB Impurity-K	16.62	15.24	16.97	15.93	16.00	16.92
8	DAB Impurity-J	4.59	4.23	4.41	4.48	4.37	4.66
9	Dabigatran Etxilate Mesylate	4.43	4.15	4.24	4.46	4.18	4.59
10	DAB Impurity-G	18.76	17.22	17.87	19.32	18.17	19.35
S. No.	Name of component	Resolution					Column change
		Idea condition	Flow decrease	Flow increase	Temp. decrease	Temp. increase	
1	DAB Impurity-A	-	-	-	-	-	-
2	DAB-2B	38.83	36.91	37.87	39.15	37.40	34.00

3	DAB Impurity-C	21.34	20.13	21.80	21.47	21.97	21.54
4	DAB Impurity-H	4.15	4.04	3.31	4.58	3.91	3.83
5	DAB Impurity-D	3.13	2.73	3.41	2.97	3.11	3.21
6	DAB Impurity-E	26.79	24.38	26.67	26.48	25.89	27.29
7	DAB Impurity-K	16.62	15.24	16.97	15.93	16.00	16.92
8	DAB Impurity-J	4.59	4.23	4.41	4.48	4.37	4.66
9	Dabigatran Etxilate Mesylate	4.43	4.15	4.24	4.46	4.18	4.59
10	DAB Impurity-G	18.76	17.22	17.87	19.32	18.17	19.35

Table 10: Robustness result in each condition

of the analytical parameters such as System suitability, Specificity, Precision, Linearity, Accuracy, Range, Intermediate precision (Ruggedness), Robustness, Solution Stability and Mobile Phase Stability. As the carryover of Dabigatran Etxilate Mesylate peak has been observed and it is not impacting the analytical results, so Dabigatran Etxilate Mesylate peak area in the blank chromatogram has to be disregarded. Thus the method stands validated and can be used for routine analysis to determine the related substances of Dabigatran Etxilate Mesylate.

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