Section A -Research paper

Identification and characterization of degradation impurities in Dabigatran etexilate drug substance

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Abstract

Dabigatran etexilate is an oral anticoagulant drug that acts as a direct thrombin inhibitor. Two impurities ranging from 0.3 to 1.0% in Dabigatran etexilate were detected by a simple isocratic reverse-phase high-performance liquid chromatography (HPLC). LC-MS was performed to identify the mass of the impurities. Based on the spectral data (NMR, and MS), the structures of these impurities were characterized as (Z)-ethyl-3-(3-(((hexyloxy) carbonyl)amino)-4-(2-((4-(N'-((hexyloxy)carbonyl)carbamimidoyl)phenyl)amino)-N-methylacetamido)-N-(pyridin-2-yl)benzamido) propanoate and 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-1*H*-benzo[*d*]imidazole-2,5-diyl-5-carbonyl))bis(pyridin-2-ylazanediyl)) dipropionate. The structure was elucidated by various 1D and 2D NMR techniques, mass spectrometry, and by comparison with the NMR data of Dabigatran etexilate.

Keywords; Dabigatran, Characterization, Spectroscopy, Structure elucidation

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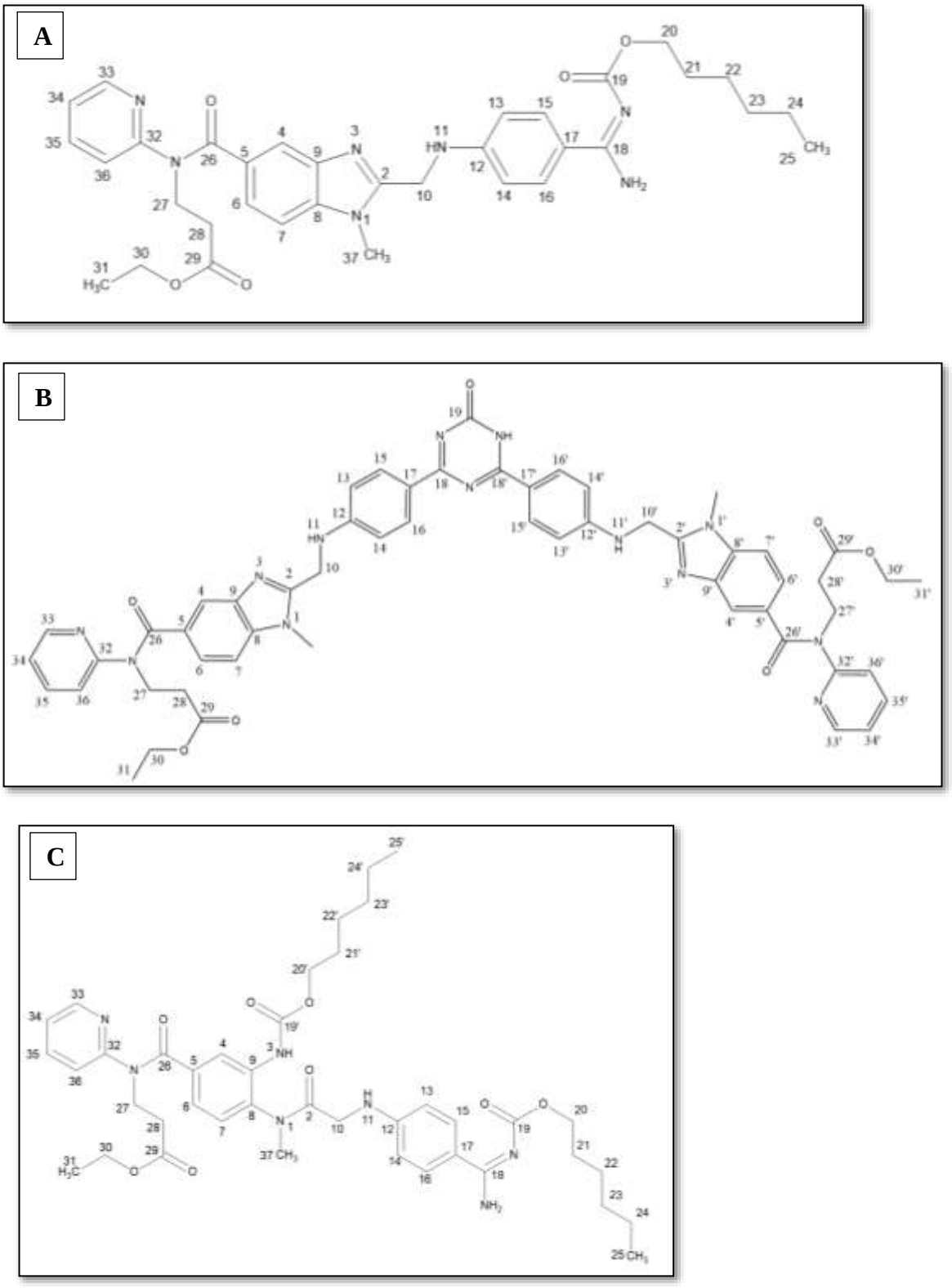

Fig-1: A) Structure of Dabigatran etexilate drug substance, B) Structure of impurity-1, C) Structure of impurity-2.

1.0 Introduction:

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

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. Some research papers have been reported in the literature for the determination of dabigatran and its impurities, but these papers were limited to Assay and Related substance method development and validation by HPLC. In this work, two unknown impurities were identified and characterized by using Mass and NMR spectroscopy data.

2.0 Material and methods:

2.1 Materials and reagents: Dabigatran etexilate API and impurity samples were obtained from the chemical research division, Dr. Reddy's Laboratories Ltd., Hyderabad, India. HPLC-grade acetonitrile was purchased from Merck India Limited. De-ionized water was prepared using a Millipore Milli-Q plus purification system. Analytical reagent grade ammonium acetate and potassium dihydrogen phosphate were purchased from Merck India Limited, and HPLC grade acetonitrile was purchased from Rankem. Dimethyl sulfoxide-d_6 and deuterium oxide D_2O (for NMR) were from CIL.

2.2 Detection by chromatography: The HPLC studies were carried out on an Agilent 1100 series quaternary pump with a degasser and as an autosampler. Inertsil ODS

C18 column (250 x 4.6 mm. 5µm,) was used for chromatographic separation. The mobile phase consists of a mixture of buffer (2mM; pH7.0 potassium dihydrogen phosphate) and Acetonitrile in the ratio of 95:5, and mobile phase B is a mixture of Acetonitrile and water in the ratio of 30:70(v/v) was used. The separation achieved with gradient program [T/A- 0.0/80, 5/80, 25/45, 45/45, 50/10, 65/10, 66/80, 75/80]. The flow rate was maintained at 1.0mL/min with UV detection at 230 nm. The column temperature was maintained at 35ºC.

2.3 Mass spectrometry: The LC-MS/MS study was carried out on an AB Sciex 4000-Q-trap spectrometer. The source voltage was kept at 4.0kV and the capillary temperature at 240°C. The sheath and auxiliary gas were selected as Nitrogen. The mass range was kept at m/z 100-1200 and 3sec scan time under positive polarity with electrospray ionization. The LC part consisted of an Agilent 1100 series quaternary pump with a degasser and an autosampler. An Inertsil ODS C18 column (250 x 4.6 mm. 5µm,) was used for chromatographic separation. The mobile phase consisted of a mixture of buffer (2mM; pH7.0 ammonium acetate) and Acetonitrile in the ratio of 95:5, and mobile phase B is a mixture of buffer and Acetonitrile in the ratio of 30:70(v/v) was used. The separation achieved with gradient program [T/A- 0.0/80, 5/80, 25/45, 45/45, 50/10, 65/10, 66/80, 75/80]. The flow rate was maintained at 1.0mL/min with UV detection at 230 nm. The column temperature was maintained at 35ºC.

2.4 HRMS DIP analysis:

The High-resolution mass spectrum of impurity-2 was recorded on the Thermo Orbitrap LC-HRMS system. The sample was introduced into the system through U-HPLC by bypassing the column. The ESI +ve ionization mass spectrum of Impurity-2 displayed the molecular mass. The proposed chemical composition by the software was within a -4.1ppm deviation.

2.5 NMR:

The NMR spectra of Dabigatran etexilate API and isolated impurities were recorded on Bruker Avance III 400MHz instrument at 25°C and operating at 400MHz for ^{1}H NMR and 100MHz for ^{13}C NMR. The analysis of API and impurity-1 were recorded using DMSO-d_6 as solvent. The ^{1}H chemical shift values

were reported on δ scale in ppm, relative to DMSO-d_6 (δ = 2.5 ppm) and the ^{13}C chemical shift values were reported relative to DMSO-d_6 (δ=39.5 ppm). DEPT, COSY, HMBC, HSQC, and D_2O Exchange, experiments were also carried out at 25°C using the same instrument. The analysis of API and Impurity-2 were recorded in $CDCl_3$ solvent. The ^1H chemical shift values were reported on the δ scale in ppm with respect to TMS (0.00ppm) and the ^{13}C chemical shift values were reported relative to $CDCl_3$ (δ 77.0ppm). The NMR analysis experiments COSY, HSQC, and HMBC were also carried out at 25°C using the same instrument.

3.0 Results and Discussion:

3.1 Detection of impurity by HPLC and LCMS

For identification of the impurities and their molecular weights LC-MS method was utilized described in section 2.3 and analyzed. The relative retention time (RRT) for impurities respective to Dabigatran Etexilate are 0.96, and 1.67 for Impurity-1 and impurity-2 respectively. The protonated molecular mass obtained from the mass spectrometer is m/z = 1008 for Impurity-1, and m/z = 774 for Impurity-2. The impurities 1 and 2 do not match with any reported impurities, so these are inferred to be new and have been given considerable attention for their structural characterization.

3.2 Elemental composition by HRMS:

The impurity-2 sample was subjected to high-resolution mass spectrometry analysis to confirm the chemical formula. Based on the isotopic ratio system has predicted the elemental composition of these impurities. Impurity-2 molecular mass was observed as [M+H] at m/z = 774.4158. The elemental composition for m/z = 774.4158 with a -4.1ppm error is $C_{41}H_{56}N_7O_8$.

Table 1: Mass for Dabigatran etexilate and impurities

Products	RRT	M.W.	Chemical formula
Dabigatran Etexilate API	1.00	627.7	$C_{34}H_{41}N_7O_5$
Impurity-1 (0.96RRT)	0.96	1007.4	$C_{55}H_{53}N_{13}O_7$
Impurity-2 (1.67RRT)	1.67	773.4	$C_{41}H_{55}N_7O_8$

3.3 Structural elucidation of impurity

The isolated impurities of Dabigatran etexilate and Dabigatran etexilate API were subjected to spectroscopic analysis such as 1D NMR (^{1}H, ^{13}C, and DEPT), 2D NMR (HSQC, HMBC, COSY), HRMS. The numbering was given to all impurities as shown in Fig.1. The NMR data of Dabigatran etexilate API and imp-1 and imp-2 are presented in Tables 2 and 3 respectively.

Impurity-1: Electrospray mass spectrum (Fig.B1) of Dabigatran etexilate 0.96 RRT impurity displayed the molecular ion at m/z=1008. This molecular weight of Dabigatran etexilate 0.96 RRT impurity is 380 mass units more than that of Dabigatran etexilate API.

The ^{1}H NMR spectrum of 0.96 RRT impurity (Fig.B2) was compared with that of Dabigatran etexilate API and found the absence of signals at δ 0.89, 1.30, 1.38, 1.68, and 4.26 ppm corresponding to hexyloxycarbonyl group and signals at δ 10.0 and 10.65 ppm corresponding to $-NH_2$ group exchangeable protons. This indicates that the hexyloxycarbonyl group is not present in impurity.

The signal at δ 12.2 ppm in the ^{1}H NMR spectrum of impurity is an extra signal integrating for one proton when compared ^{1}H NMR spectrum of Dabigatran etexilate.

In the ^{1}H NMR spectrum of Dabigatran etexilate API, the proton count of exchangeable proton and the proton count of the Dabigatran etexilate moiety indicates the ratio to be 1:1. On the other hand, the ratio is 1:2, when the proton count of exchangeable proton at δ 12.15 ppm is compared with that of Dabigatran moiety, in the ^{1}H NMR spectrum of Dabigatran etexilate impurity. This observation is an indication of a dimer formation.

It is interesting to note that the signals due to the proton connected to C15 and C16 have moved from δ 7.65 ppm in Dabigatran etexilate API to δ 8.20 ppm in the impurity. This could be due to the change in the substitution at the C17 position.

The ^{13}C NMR spectrum of Impurity-1 (Fig. B3), when compared with the Dabigatran etexilate API ^{13}C NMR spectrum the quaternary carbon signal

(Position-18) at δ 163.4 ppm in Dabigatran etexilate is shifted to δ 152.5 ppm as a broad signal, which indicates the change in the chemical environment.

The gHMBC spectrum for impurity showed the connectivity for the aromatic protons at δ 8.20 ppm (Position 15, 16) with the ^{13}C signal at δ 152.5 ppm supports the above observation.

Impurity-2: The ESI +ve ionization mass spectrum of Dabigatran 1.67RRT impurity displayed the protonated molecular ion at m/z = 774. This has 146 amu more than the Dabigatran etexilate molecular mass. This matches with the additional substitution of hexyl formate and hydroxyl group to the Dabigatran etexilate structure. The HRMS spectrum (Fig. C2) confirms the molecular formula of Dabigatran 1.67RRT impurity as $C_{41}H_{55}N_7O_8$.

In the proton NMR spectrum, (Fig. C3) of the 1.67RRT impurity additional signal counts were observed at 0.85, 1.3, 1.7 ppm, and 4.1ppm when compared with Dabigatran etexilate API proton NMR spectrum. These were attributed to the additional one methyl and five methylene groups of hexyl formate.

The signal for the methylene group connected to secondary amine (position no. 10) Dabigatran etexilate API shifted from 4.6ppm to 3.4ppm in the 1.67RRT impurity. When comparing the ^{13}C NMR spectrum (Fig. C4) of 1.67RRT impurity with that of Dabigatran etexilate API ^{13}C NMR spectrum, the quaternary carbon signal (position no 2) is observed at 151ppm and 169 ppm in API and impurity respectively. This indicates that quaternary carbon at position 2 in Dabigatran etexilate API is converted into the C=O group.

In Dabigatran etexilate carbon signal for methine carbons at position 6 was observed at 108ppm, whereas in impurity same carbon signal was observed at 124ppm. The proton signal for position 4 is observed at 7.6 ppm in Dabigatran etexilate. The same was shifted to 8.6ppm in impurity. These chemical shift variations lend support to the cleavage of the imidazole ring and subsequent substitution of hexyl formate at position 3.

The HMBC spectrum (Fig. C7) of 1.67RRT impurity displayed the correlations of quaternary carbons at 130ppm (position 8) with methine protons at 8.32, 7.05, 6.98ppm (positions 4, 6, and 7 respectively), methyl proton signal at 3.25ppm

(position-37) and one exchangeable proton signal at 6.85ppm. This supports the imidazole ring opening at the C2-N3 bond and the formation of an amide bond between hexyl formate carbonyl carbon and nitrogen at position 3.

Table 2: Structural assignments for Dabigatran Etexilate mesylate and impurity-1 (DMSO-d_6)

API			Impurity-1		
Position[#]	δ (ppm)^{1}H	^{13}C	Position[#]	δ (ppm)^{1}H	^{13}C
2	-	153.2	2, 2’	-	153.5
4	7.5 (1H, s)	122.9	4, 4’	7.45 (2H, s)	119.5
5	-	129.5	5, 5’	-	130.2
6	7.4 (1H, d)	109.6	6, 6’	7.4 (2H, d)	109.5
7	7.2 (1H, d)	121.3	7, 7’	7.2 (1H, d)	122.8
8	-	137.9	8, 8’	-	137.2
9	-	137.9	9, 9’	-	137.2
10	4.65 (2H, br)	39.9	10, 10’	4.65 (4H, br)	40.0
11	7.65 (NH, br)	-	11, 11’	7.25 (2NH, br)	-
11(CH3SO3H)	2.3 (3H, s)	40.1	-	-	-
11(CH3SO3H)	10.0 (OH, br)	-	-	-	-
12	-	140.3	12, 12’	-	140.8
13	6.9 (1H, d)	112.4	13, 13’	6.8 (2H, d)	111.7
14	6.9 (1H, d)	111.7	14, 14’	6.8 (2H, d)	111.7
15	7.65 (1H, d)	131.4	15, 15’	8.2 (2H, d)	129.3
16	7.65 (1H, d)	131.4	16, 16’	8.2 (2H, d)	129.3
17	-	153.7	17, 17’	-	152.5
18	-	163.4	18, 18’	-	152.8
18(NH2)	10.65	-	18(NH)	12.15 (NH, br)	-
	11.90	-	-	-	-
19	-	154.1	19	-	156.7
20	4.26 (2H, m)	66.9	-	-	-
21	1.68 (2H, m)	27.9	-	-	-
22	1.38 (2H, m)	24.8	-	-	-
23	1.3 (2H, m)	30.0	-	-	-
24	1.3 (2H, m)	22.0	-	-	-
25	0.89 (3H, t)	13.9	-	-	-
26	-	171.0	26, 26’	-	171.0
27	4.2 (2H, m)	44.3	27, 27’	4.2 (2H, m)	44.3
28	2.7 (2H, t)	33.3	28, 28’	2.7 (4H, t)	33.0
29	-	170.2	29, 29’	-	170.3
30	4.0 (2H, q)	60.0	30, 30’	4.0 (4H, q)	60.0
31	1.15 (3H, t)	13.9	31, 31’	1.2 (6H, t)	13.9
32	-	155.9	32, 32’	-	156.0
33	8.4 (1H, d)	148.7	33, 33’	8.4 (2H, d)	148.6
34	7.2 (1H, t)	122.1	34, 34’	7.1 (2H, t)	121.2
35	7.56 (1H, t)	137.1	35, 35’	7.55 (2H, t)	137.8
36	6.9 (1H, d)	119.3	36, 36’	6.9 (2H, d)	122.1
37	3.8 (3H, s)	30.8	37, 37’	3.8 (6H, s)	33.0

[#] Refer to the structural formula in Fig-1 for numbering
d-doublet, t-triplet, m-multiplet, br-broad.

Table 3: Structural assignments for Dabigatran Etexilate and impurity-2 ($CDCl_3$)

API			Impurity-2		
Position[#]	δ (ppm)^{1}H	^{13}C	Position[#]	δ (ppm)^{1}H	^{13}C
2	-	150.5	2	-	169.4
4	7.65 (1H, s)	123.8	4	8.4 (1H, s)	120.9
5	-	130.0	5	-	135.1
6	7.0 (1H, d)	108.8	6	7.0 (1H, d)	124.1
7	7.0 (1H, d)	121.0	7	7.0 (1H, d)	122.2
8	-	137.3	8	-	130.7
9	-	137.3	9	-	138.2
10	4.65 (2H, br)	40.7	10	3.35 (Ha, dd)	44.9
				3.55 (Ha, dd)	-
11	5.4 (NH, br)	-	11	5.04 (NH, br)	-
12	-	152.3	12	-	150.1
13	6.65 (1H, d)	112.2	13	6.35 (1H, d)	111.9
14	6.65 (1H, d)	112.2	14	6.35 (1H, d)	111.9
15	7.7 (1H, d)	129.1	15	7.7 (1H, d)	129.2
16	7.7 (1H, d)	129.1	16	7.7 (1H, d)	129.2
17	-	120.3	17	-	122.0
18	-	167.6	18	-	167.2
18(NH2)	10.65	-	18(NH2)	9.5	-
-	11.90	-		-	-
19	-	165.2	19	-	164.3
-	-	-	19’	-	153.0
20	4.15 (2H, m)	65.4	20, 20’	4.15 (4H, m)	65.7
21	1.75 (2H, m)	28.9	21	1.7 (2H, m)	28.7
-	-	-	21’	1.65 (2H, m)	28.8
22	1.4 (2H, m)	25.2	22	1.4 (2H, m)	25.6
-	-	-	22’	1.3 (2H, m)	25.6
23	1.3 (2H, m)	29.8	23, 23’	1.3 (4H, m)	28.8
24	1.3 (2H, m)	22.6	24, 24’	1.3 (4H, m)	22.6
25	0.89 (3H, t)	14.1	25, 25’	0.9 (6H, t)	14.1
26	-	171.7	26	-	169.6
27	4.2 (2H, m)	44.3	27	4.4 (2H, t)	44.8
28	2.7 (2H, t)	33.3	28	2.8 (2H, t)	33.1
29	-	170.2	29	-	171.6
30	4.4 (2H, m)	60.5	30	4.1(2H, m)	60.6
31	1.2 (3H, t)	14.0	31	1.25 (3H, t)	14.0
32	-	156.1	32	-	155.4
33	8.4 (1H, d)	148.9	33	8.4 (1H, d)	149.1
34	7.2 (1H, t)	123.1	34	7.2 (1H, t)	121.8
35	7.35 (1H, t)	141.0	35	7.55 (1H, t)	137.6
36	6.7 (1H, d)	122.4	36	7.1 (1H, d)	127.6
37	3.6 (1H, s)	31.6	37	3.2 (1H, s)	36.2

[#] Refer to the structural formula in Fig-1 for numbering
d-doublet, t-triplet, m-multiplet, br-broad.

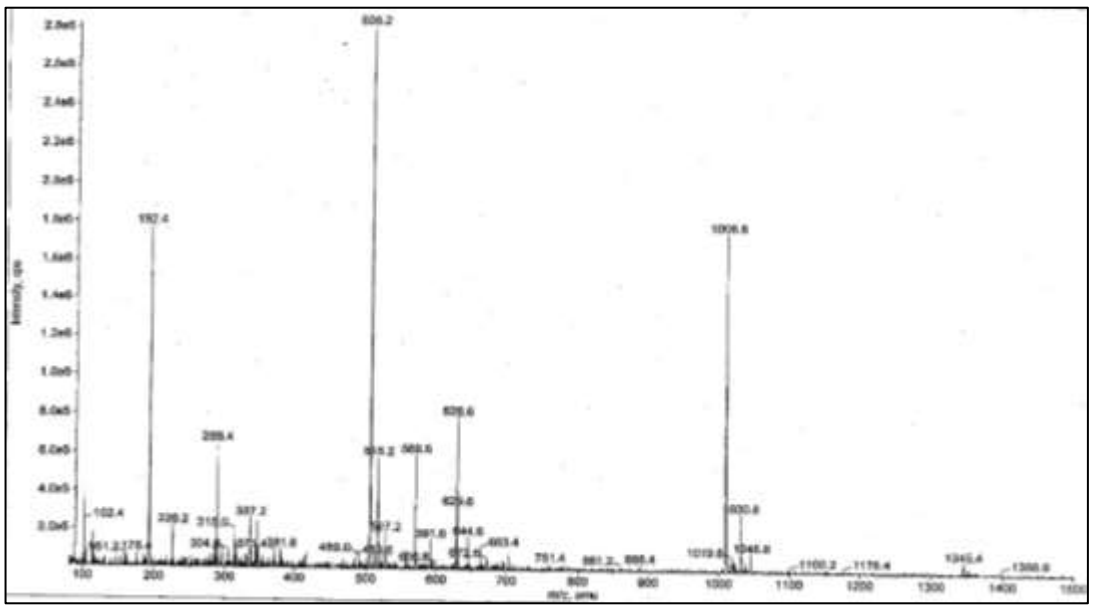

Fig-B1: ESI +ve Mass spectrum of Dabigatran etexilate impurity-1.

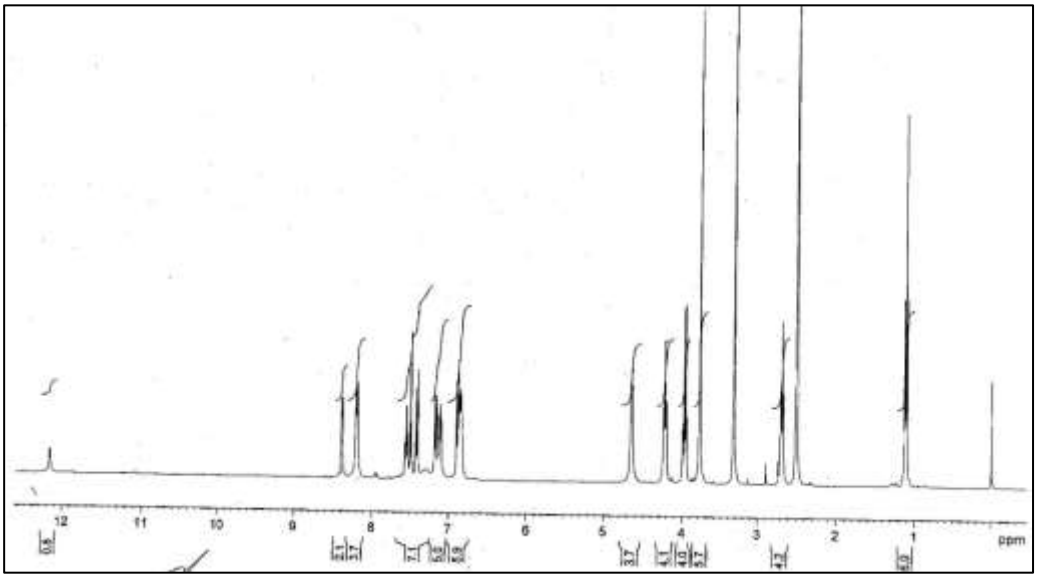

Fig-B2: ^{1}H NMR spectrum of Dabigatran etexilate impurity-1 in DMSO-d_6.

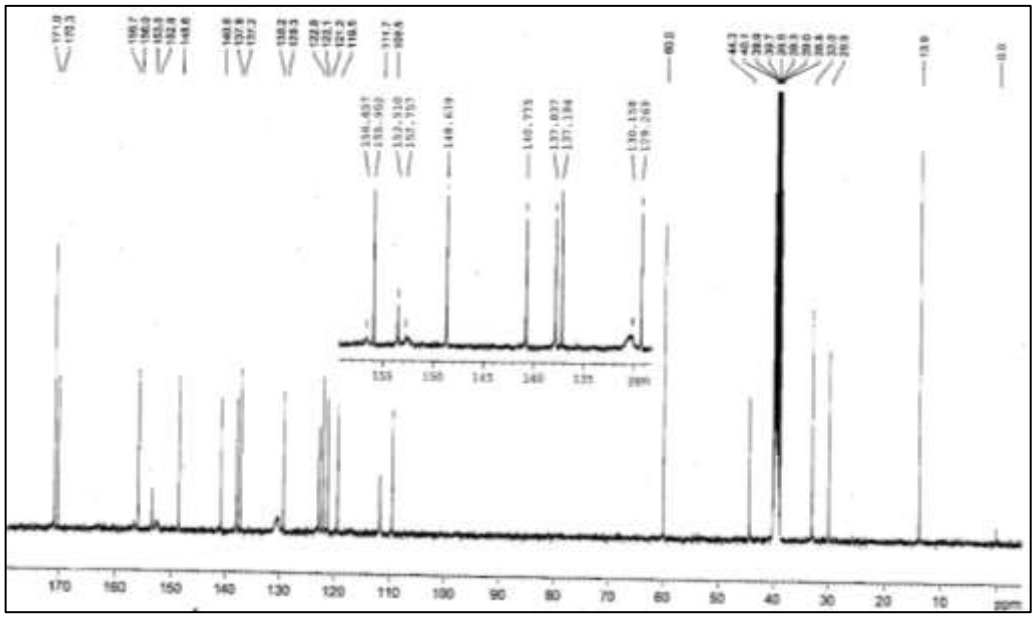

Fig-B3: ^{13}C NMR spectrum of Dabigatran etexilate impurity-1 in DMSO-d_6.

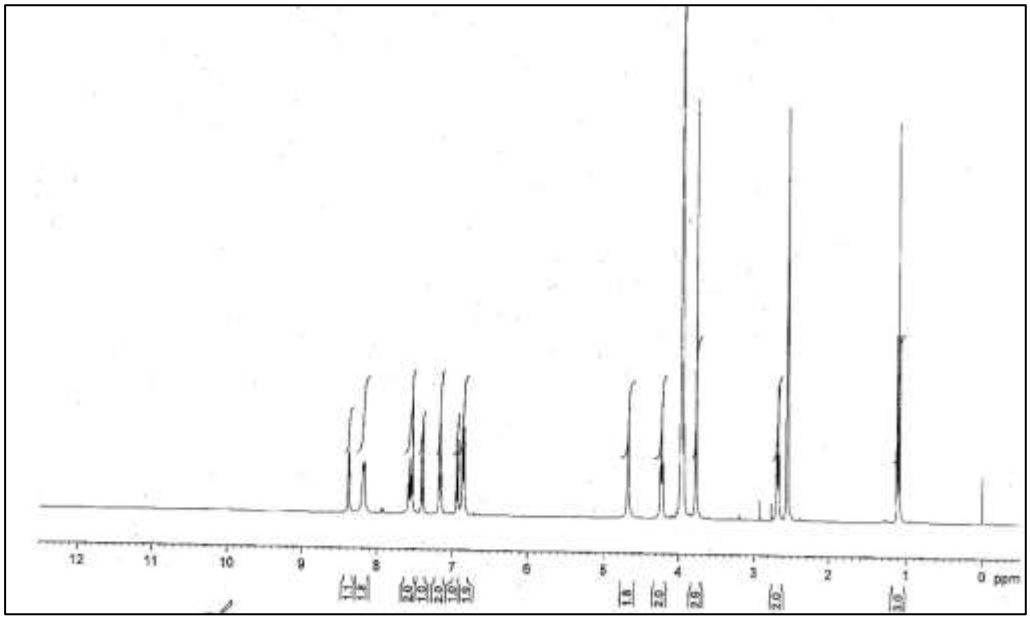

Fig-B4: D_2O NMR spectrum of Dabigatran etexilate impurity-1 in DMSO-d_6.

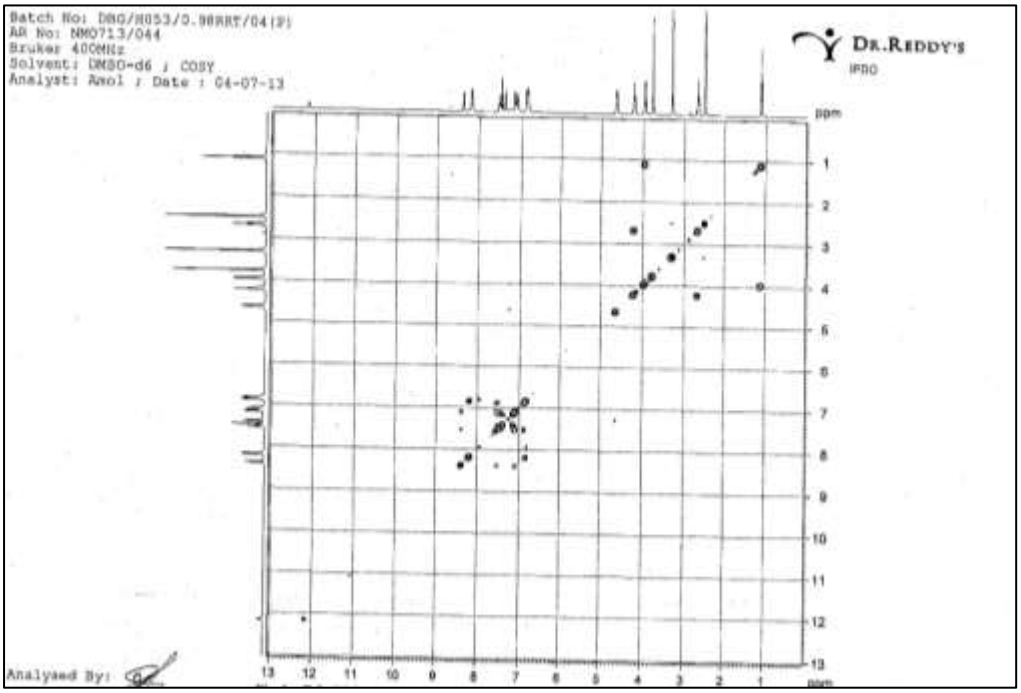

Fig-B5: COSY NMR spectrum of Dabigatran etexilate impurity-1 in DMSO-d_6.

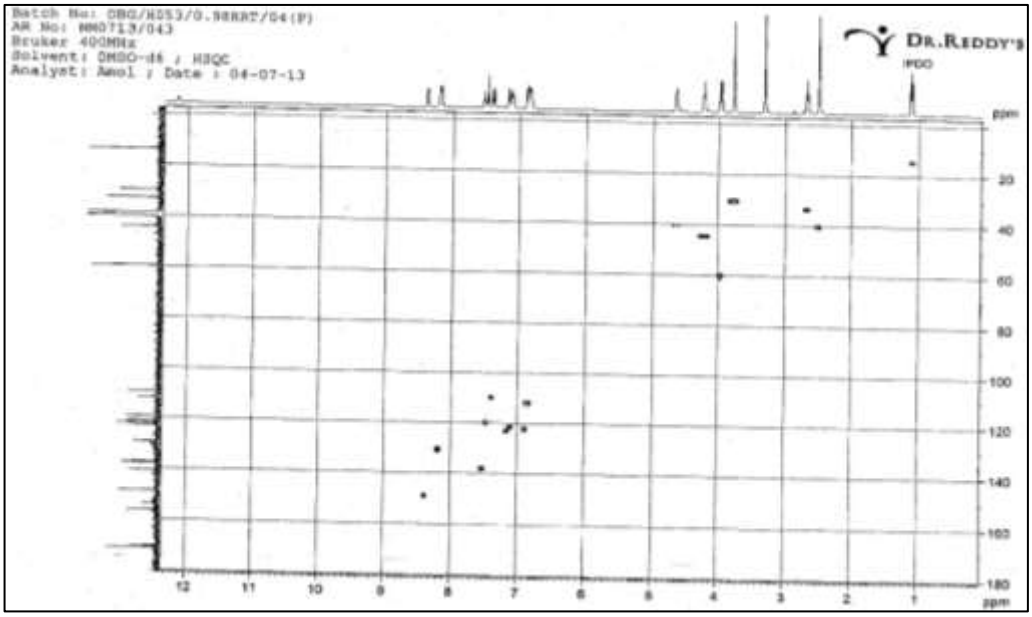

Fig-B6: gHSQC NMR spectrum of Dabigatran etexilate impurity-1 in DMSO-d_6.

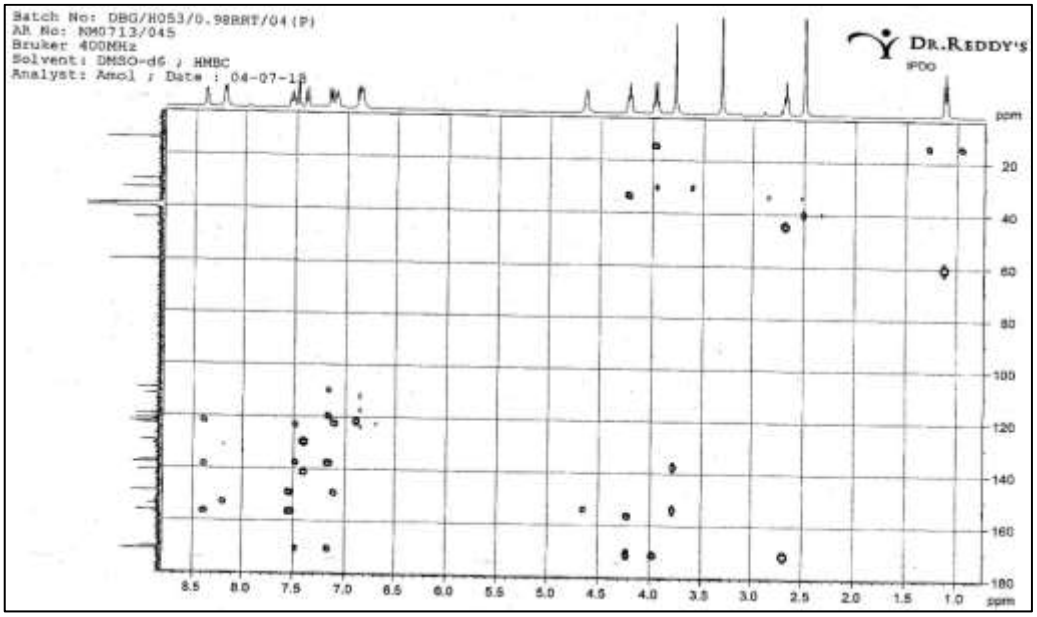

Fig-B7: HMBC NMR spectrum of Dabigatran etexilate impurity-1 in DMSO-d_6.

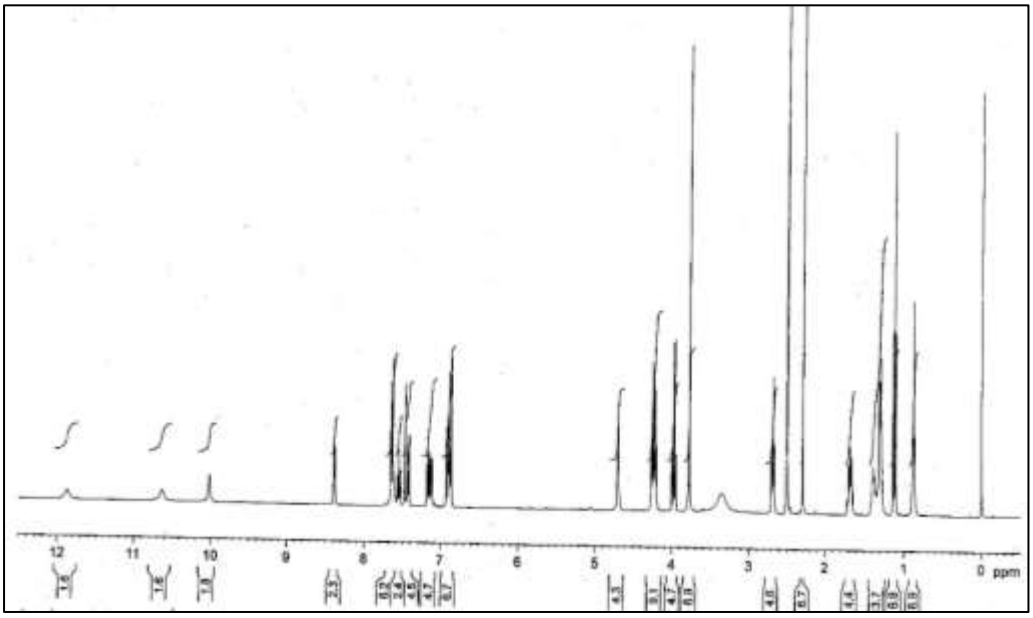

Fig-B8: ^{1}H NMR spectrum of Dabigatran etexilate mesylate API in DMSO-d_6

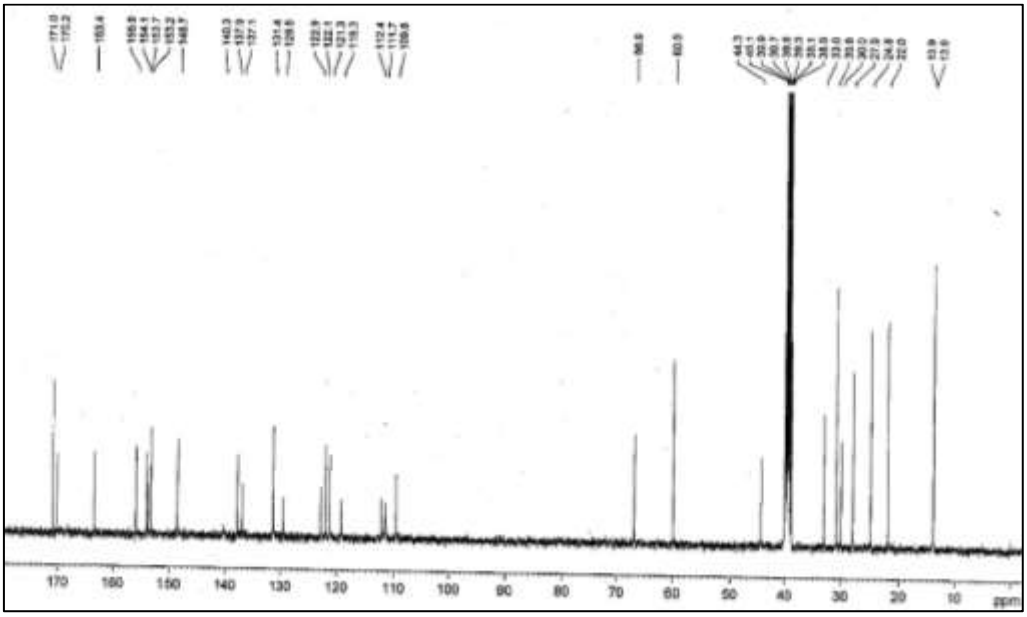

Fig-B9: ^{13}C NMR spectrum of Dabigatran etexilate mesylate API in DMSO-d_6

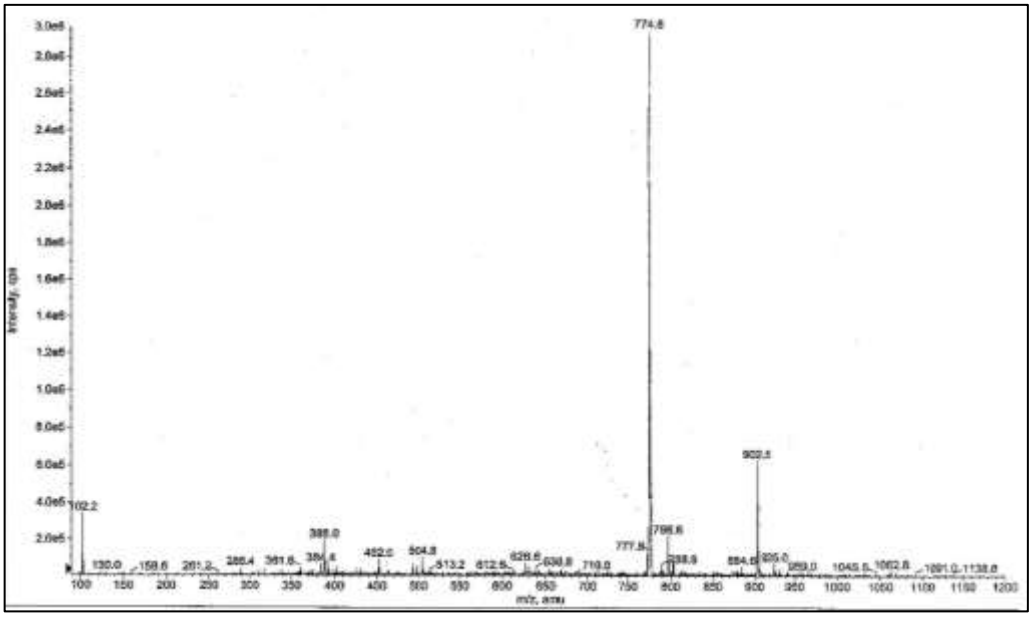

Fig-C1: ESI +ve Mass spectrum of Dabigatran etexilate impurity-2.

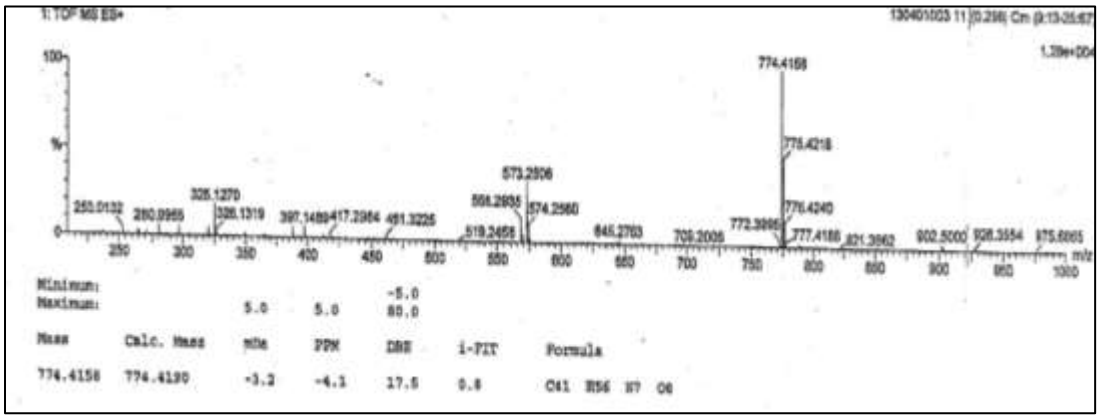

Fig-C2: HRMS Mass spectrum of Dabigatran etexilate impurity-2.

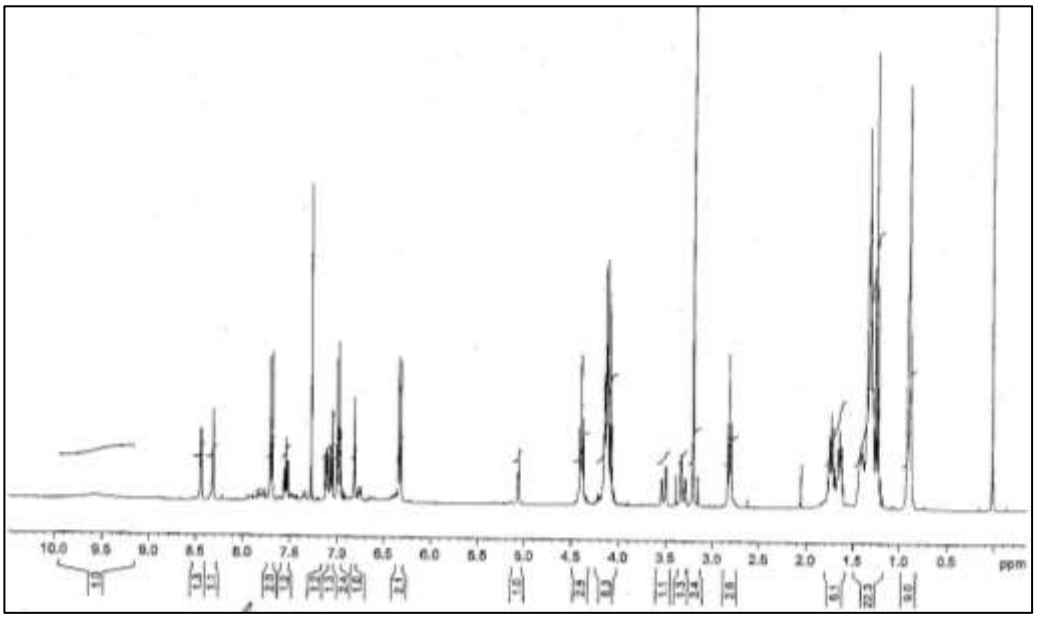

Fig-C3: ^{1}H NMR spectrum of Dabigatran etexilate impurity-2 in $CDCl_3$.

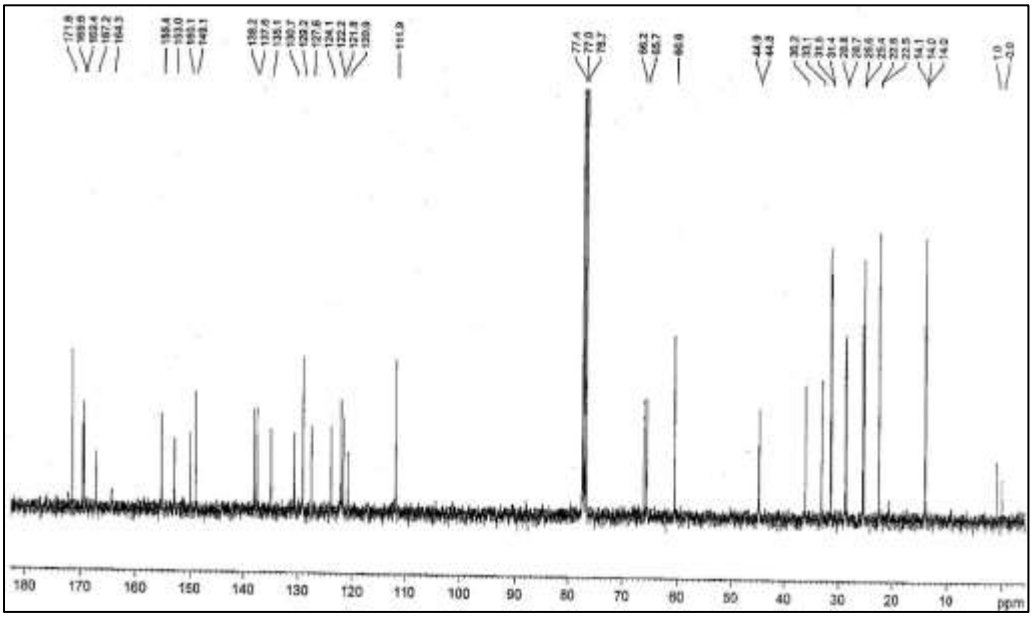

Fig-C4: ^{13}C NMR spectrum of Dabigatran etexilate impurity-2 in $CDCl_3$.

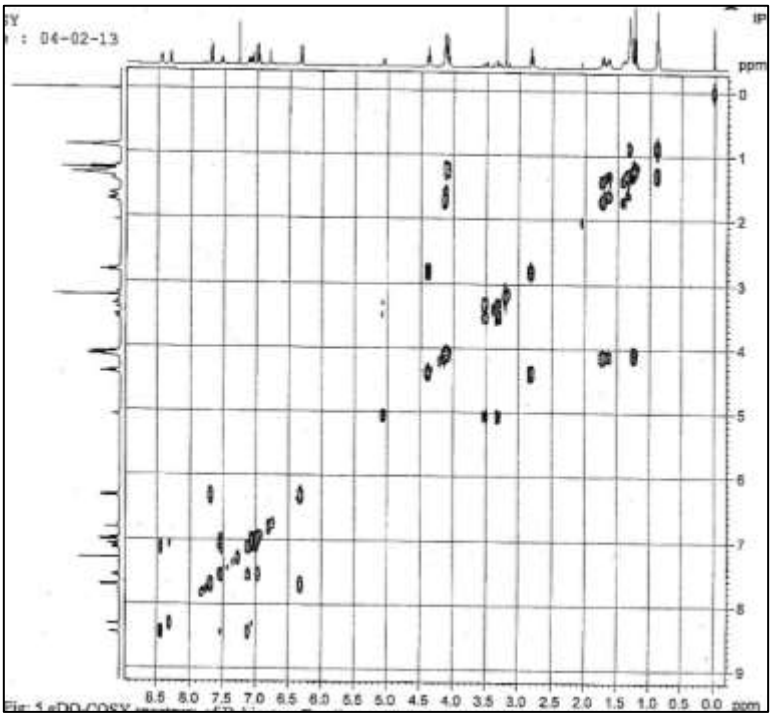

Fig-C5: COSY NMR spectrum of Dabigatran etexilate impurity-2 in $CDCl_3$.

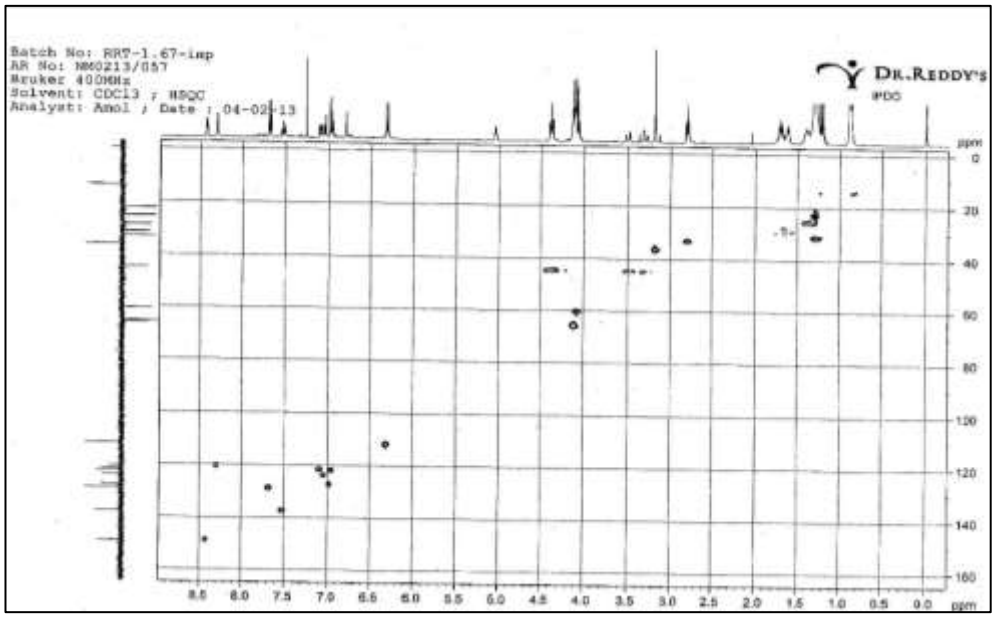

Fig-C6: gHSQC NMR spectrum of Dabigatran etexilate impurity-2 in $CDCl_3$.

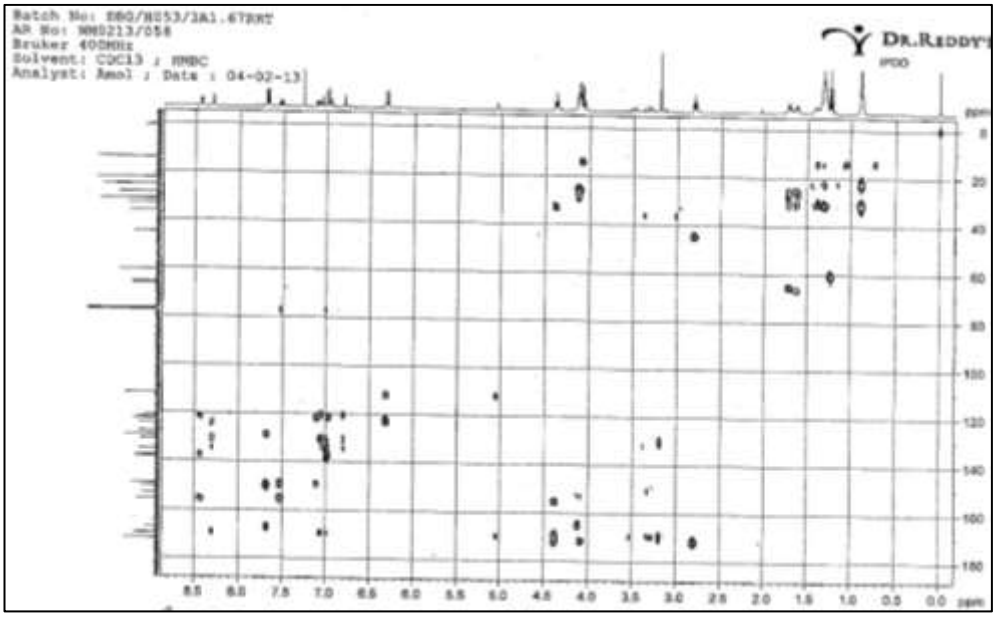

Fig-C7: HMBC NMR spectrum of Dabigatran etexilate impurity-2 in $CDCl_3$.

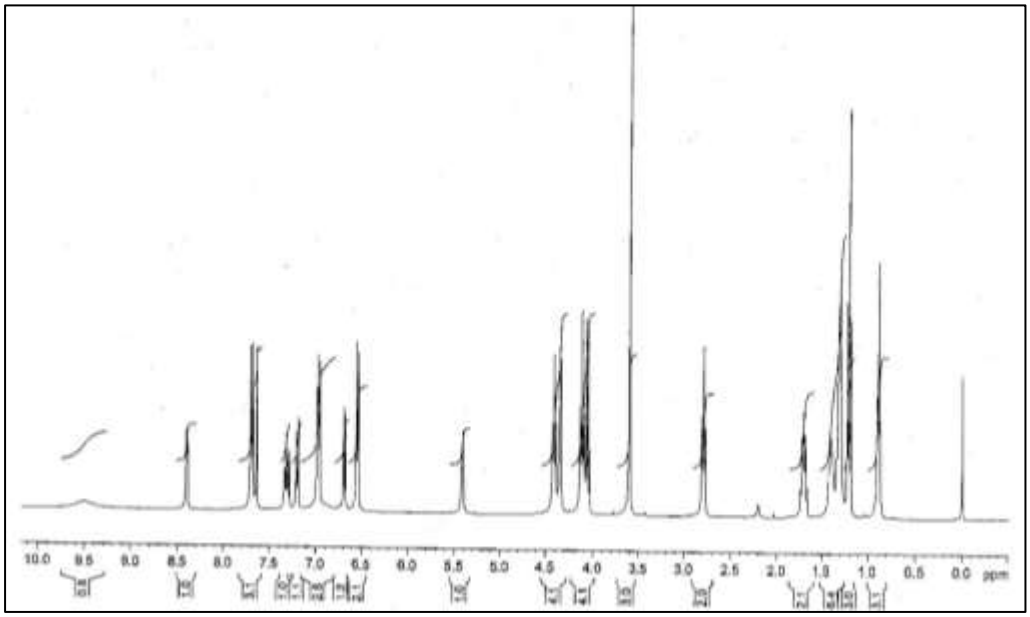

Fig-C8: ^{1}H NMR spectrum of Dabigatran etexilate API in $CDCl_3$.

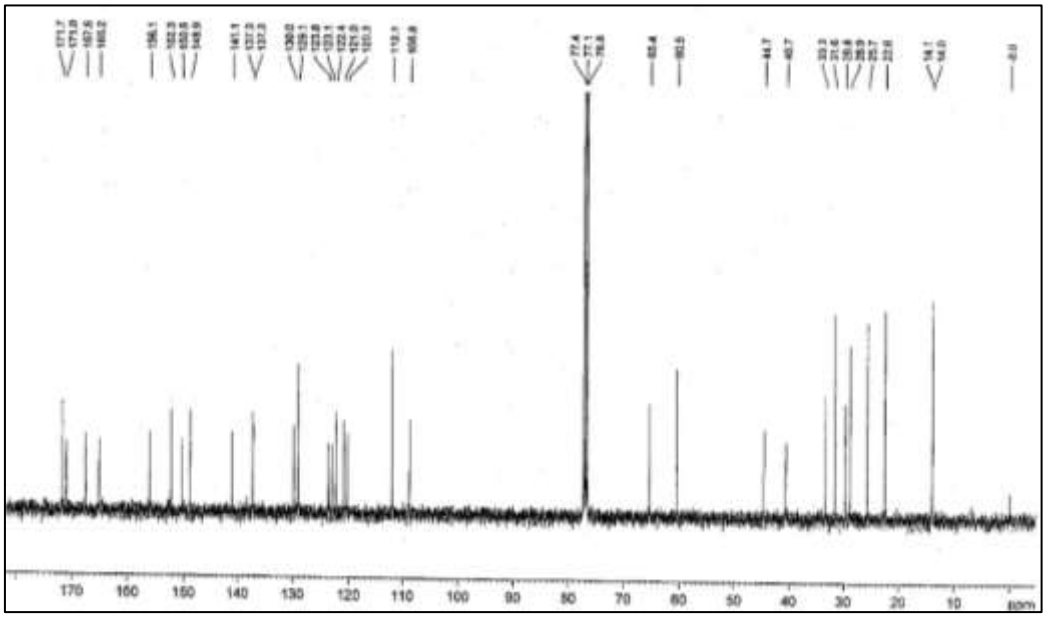

Fig-C9: ^{13}C NMR spectrum of Dabigatran etexilate API in $CDCl_3$.

4.0 Conclusion

Two new process-related impurities in the preparation of Dabigatran etexilate drug substance were identified and the structures were elucidated by various techniques HRMS, LC-MS, 1D NMR (^{1}H, ^{13}C, and DEPT), 2D NMR (COSY, HSQC, HMBC). The proposed chemical structures of impurities were confirmed and identified the root of synthesis of these impurities. Based on this information impurities were controlled in the root of synthesis for the Dabigatran etexilate drug substance and a pure compound was obtained.

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