

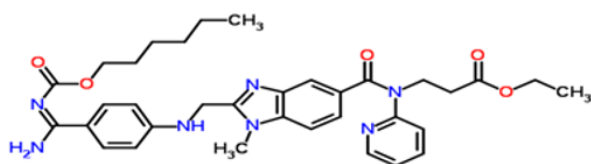
LC-MS method for Analysis of Dabigatran and its Impurities

Basima Arous* and Mohammad Amer Al-Mardini*Department of Pharmaceutical Chemistry and Quality Control, Faculty of Pharmacy, Damascus University, Damascus, Syria****Corresponding Author:** Basima Arous, Department of Pharmaceutical Chemistry and Quality Control, Faculty of Pharmacy, Damascus University, Damascus, Syria**Received:** February 15, 2019; **Published:** April 25, 2019**Abstract**

Dabigatran is a novel anticoagulant drug acting as a direct and reversible thrombin inhibitor. The purpose of this work was to develop a sensitive and validated LC-MS method for the analysis of Dabigatran and estimation of its main three impurities in API and pharmaceutical dosage forms. The analysis were carried out on a Shimadzu Shim-pack XR-ODS II column (100 x 3.0 mm, 2.2 μ m particle size), with a mobile phase containing water with 0.1% of formic acid as mobile phase A and acetonitrile as mobile phase B in gradient program at a flow rate of 0.3ml/min, the column oven temperature was at 30°C, the UV detection was at 225 nm and the impurities were characterized by ESI-MS. The method was validated according to USP 35 guideline recommendations and to the ICH guidelines for validation. The linearity, selectivity, accuracy, and robustness of the developed method showed acceptable values. The method is suitable for practical routine analysis of API and pharmaceutical dosage forms, and it was applied to analyze Pradaxa® the brand. All the analysis results were acceptable according to the pharmaceutical requirements, but impurity D which was found in Pradaxa® and in all analyzed API exceeded the impurities acceptable limits.

Keywords: Dabigatran; Impurities; HPLC; LC-MS; Validation**Introduction**

Dabigatran etexilate (Figure 1), chemically known as (ethyl 3-[[[2-[[[4-[[[hexyloxy] carbonyl] carbamimidoyl] phenyl] amino] methyl]-1-methyl-1H-benzimidazol-5-yl] carbonyl] (pyridin-2-yl) amino] propanoate), is a pro-drug, rapidly converted to Dabigatran after oral administration. It is a novel anticoagulant drug invented and manufactured by Boehringer Ingelheim, acting as a direct, selective and reversible thrombin inhibitor. It is prescribed for the prevention of stroke and systemic thromboembolism after elective hip and knee replacement in patients with nonvalvular atrial fibrillation [1,2].

**Figure 1:** Structure of the Dabigatran etexilate [3].Molecular Formula: C₃₄H₄₁N₇O₅

Molecular Weight: 627.75

Very few methods appeared in the literature for the determination of Dabigatran etexilate and its related substances (impurities) in active pharmaceutical ingredient (API) and pharmaceutical dosage forms. One UPLC MS/MS method has been reported for the quantitative analysis of Dabigatran etexilate in human plasma for therapeutic monitoring [4], other LC-MS method was reported for the determination of Dabigatran etexilate, intermediate metabolite and dabigatran in rat plasma and its application to pharmacokinetic study [5]. Also there are few RP-HPLC methods reported in the literature that can estimate Dabigatran etexilate and its process related impurities in (API) and pharmaceutical dosage forms [6-9]. Furthermore, there are several stability-indicating methods which have been reported for the stability profile of Dabigatran etexilate and determination of its potential degradation products [9-17].

Moreover, there is no HPLC/LC-MS method reported in the literature that can estimate Dabigatran etexilate and adequately separate its process related impurities. In addition, Dabigatran etexilate is not yet official in any of the pharmacopoeia [18-20]. Therefore, it is necessary to develop a sensitive HPLC method for analysis of Dabigatran etexilate and estimation of its related substances in API and pharmaceutical dosage forms. The aim of this research work was to develop a precise, sensitive and accurate HPLC method for the determination of three process related impurities of Dabigatran

etexilate, then the three impurities were detected and identified by ESI-MS. The developed method was successfully validated according to the USP 35 Validation of Compendial Procedures [19] and ICH guidelines [21]. Then the method was applied to analyze Pradaxa® the brand.

The three Dabigatran etexilate related impurities are:

- Imp A: Ethyl 3-(2-((4-carbamimidoylphenylamino)methyl)-1-methyl-N-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamido) propanoate.
- Imp B: 3-[[[2-[[[4-Cyanophenyl]amino]methyl]-1-methyl-1H-benzimidazol-5-yl]carbonyl]pyridin-2-ylamino]propionic acid ethyl ester.
- Imp C: Ethyl-3-(1-[2-[[[4-[amino{[(methoxy)carbonyl]imino}]methyl]phenyl]amino]methyl]-1-methyl-1H-1,3-benzodiazol-5-yl]-N-(pyridin-2-yl)formamido) propanoate.

Materials and Methods

Chemicals and reagents

Dabigatran etexilate standard was purchased from Megafine Pharma (P) LTD. Impurities (A,B and C) standards were purchased from Megafine Pharma (P) LTD. Active pharmaceutical ingredients were obtained from Megafine Pharma (P) Ltd and from Shandong Rongyuan Pharmaceuical Co, Ltd. HPLC acetonitrile and Formic acid were purchased from SIGMA-ALDRICH®. HPLC water was obtained by Siemens Water Technologies LaboStar. Pradaxa® capsules were purchased from Boehringer Ingelheim Pharma GmbH and Co.KGIngelheim am Rhein Germany.

Instruments

The chromatographic analysis was performed with SHIMADZU LC prominence system (Shimadzu, Japan) provided with UV-Vis Detector SPD 20A, MS Detector 2020, two pumps A and B: LC/20AD, column oven CTO-20A, manual injector and with Shim-pack XR-ODS II (100 x 3.0 mm, 2.2µm particle size), System control and data analysis were carried out using LabSolutions CS (Schimadzu, Japan). KNAUER HPLC Smartline system with PDA detector (Germany). Sartorius sensitive analytical balance (sensitivity of 10-4g). JEKEN Digital Ultrasonic Cleaner. Bechers, Volumetric flasks, Micropipettes and Glassware of different volumes from Marienfeld Company. Filters PVDF 0.45µm for HPLC purchased from TEKNOK-ROMA.

Chromatographic conditions

The chromatographic separation was performed on Shim-pack XR- ODS II (100 x 3.0 mm, 2.2µm particle size) at a column temperature of 30°C, The mobile phase A was water with 0.1% of formic acid, while the mobile phase B was acetonitrile, the gradient program of the mobile phase was set as [Time(min)/Pump B Value(%)] [0.01/15, 17/60 and 25/80], The mobile phase was filtered using 0.45 µm disposable filter, and degassed by ultrasonic vibration prior to use. The flow rate was 0.3 ml/min. The injection volume was 20 µL and the detection was carried out at 225nm. Water and acetonitrile 50:50 (v/v) was used as a diluent. The analysis

was performed in positive electro-spray/positive ionization mode ESI+, the ion source voltage was 5000 V, the source temperature was 450 °C, and the curtain gas flow was 15 psi.

Preparation of solutions

Preparation of stock solutions

Stock solutions of Dabigatran etexilate was prepared by dissolving 200 mg of Dabigatran etexilate standard in diluent and made up to volume to obtain a concentration of 2.0 mg/ml. Stock solutions of Impurities A,B and C were prepared by dissolving each Impurity standard in diluent to obtain a concentration of 0.2 mg/ml.

Preparation of the standard mixture solution of Dabigatran etexilate and Impurities A,B and C

Standard mixture solution of Dabigatran etexilate and Impurities A,B and C was prepared from the previous stock solutions to obtain a solution containing 1 mg/ml Dabigatran etexilate and 0.01mg/ml of each Impurity.

Preparation of Pradaxa® solution

From a quantity of the mixed contents of 20 capsules containing 150 mg of Dabigatran etexilate, Pellets equivalent to 10 mg of the drug were dissolved and made up to volume 10 ml with diluent to make 1 mg/ml solution. This solution was filtered using 0.45 µm disposable filter.

Preparation of solutions for validation study

Linearity was studied across concentration range 200–1000 µg/ml of Dabigatran etexilate and 2-10 µg/ml of each Impurity. They were prepared from the standard mixture solution of Dabigatran etexilate and Impurities A,B and C. For accuracy nine samples were divided into three groups containing respectively 50%, 100% and 150% of Dabigatran etexilate and Impurities A,B and C (500/5, 1000/10, 1500/15, Dabigatran etexilate/each impurity, µg/ml), and the recovery was calculated. For precision six replicates of standard mixture solution of Dabigatran etexilate and Impurities A,B and C were done and the repeatability of the method was checked, the RSD% was calculated, and the intermediate precision of the assay method was evaluated on different day by different analyst using an instrument located within the same laboratory. The limits of detection (LOD) and quantification (LOQ) were determined at a signal-to-noise ratio of 3 : 1 and 10 : 1, respectively. The robustness study was carried out by analyzing three samples of standard mixture solution of Dabigatran etexilate and Impurities A,B and C (1.0 mg/ml and 0.01 mg/ml of each impurity respectively) after adjusting the flow rate to 0.2ml/min, 0.3ml/min and to 0.4ml/min whereas all other mobile phase components were held constant as described above.

Results

Method development

The main target of the chromatographic method is to achieve the separation of impurities from Dabigatran etexilate in the purchased active pharmaceutical ingredient (API). According to the Dabigatran etexilate nature and solubility tests (it is practically

insoluble in water, slightly soluble in organic solvents and freely soluble in DMSO), it was decided to use the mixture of acetonitrile and water with 0.1% of formic acid in different proportion as a mobile phase for the separation of Dabigatran etexilate and its impurities because the separation and the peaks shape were better than by using methanol and water. And as a stationary phase the HPLC columns used in this study were as follows:

- Shim-pack XR- ODS II (100 x 3.0 mm, 2.2µm particle size).
- C18 column (250 x 4.6 mm, 5µm particle size).
- C18 column (150 x 4.6 mm, 5µm particle size).

When using the C18 columns with 5µm particle size the impurities were merged, and the peaks shape were tailing and fronting (Figure 2), therefore it was decided to use the ODS II column (100 x 3.0 mm, 2.2µm particle size).

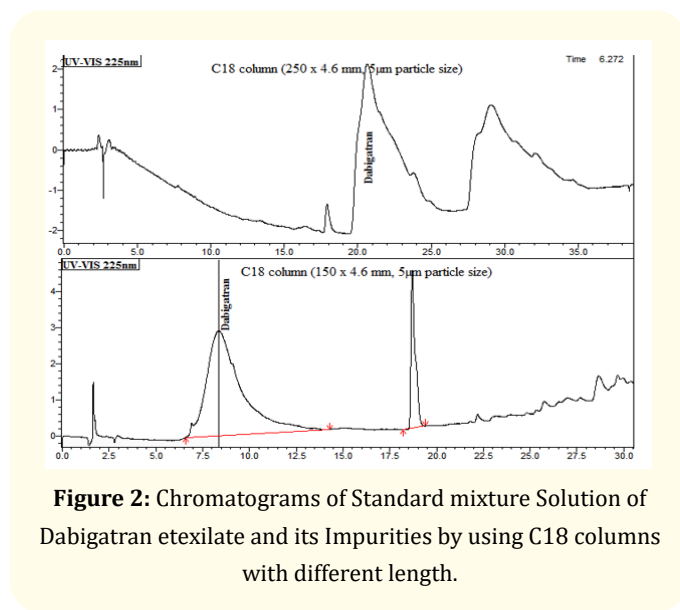


Figure 2: Chromatograms of Standard mixture Solution of Dabigatran etexilate and its Impurities by using C18 columns with different length.

More than fifty isocratic and gradient programs were tried and the best separation of Dabigatran etexilate and its impurities was achieved by using the mobile phase Acetonitrile: water with 0.1% of formic acid as described previously, and by using the column temperature 30°C where the column pressure was 137 bar and the retention time for Dabigatran etexilate was 8.984 min. The standard mixture solution of Dabigatran etexilate and Impurities A,B and C showed maximum absorbance at 225 nm after scanning between 200-400 nm (Figure 3). The increasing flow-rate from 0.1ml/min to 0.5ml/min showed a decrease in the retention time. Sufficient flow rate of 0.3ml/min was chosen to avoid overlap between peaks and the loss of its acceptable resolution values (Figure 4).

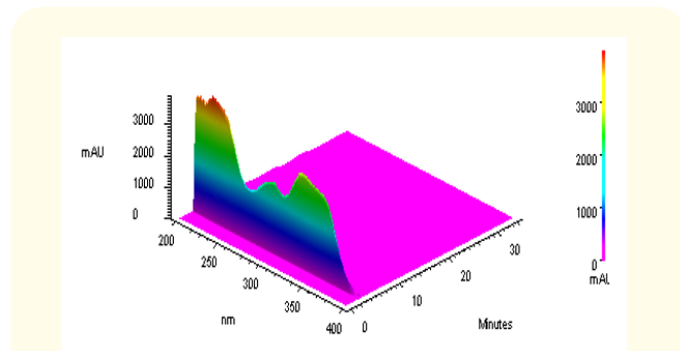


Figure 3: UV spectrum of standard solution of Dabigatran etexilate By Using HPLC-PDA.

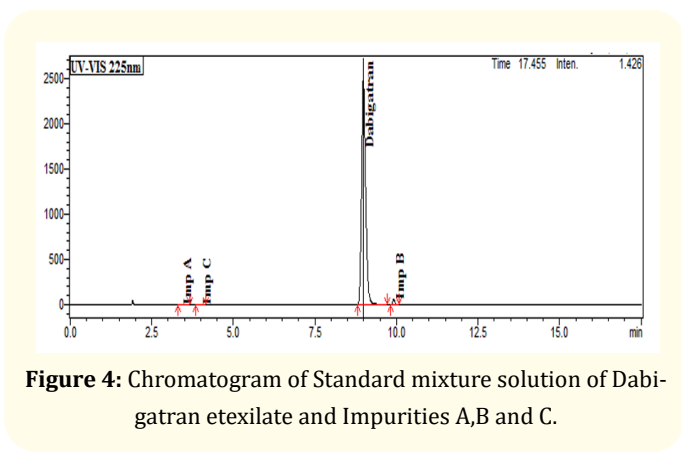


Figure 4: Chromatogram of Standard mixture solution of Dabigatran etexilate and Impurities A,B and C.

Samples of Dabigatran etexilate API test results and Capsules dosage forms test results

Two samples of Dabigatran etexilate (API) from different sources, one Dabigatran etexilate working standard and Pradaxa® capsules were analyzed according to this new chromatographic method, the concentrations were calculated from the peak areas, the impurities were successfully separated from Dabigatran etexilate, and the results were shown in (Figure 5) and Table 1.

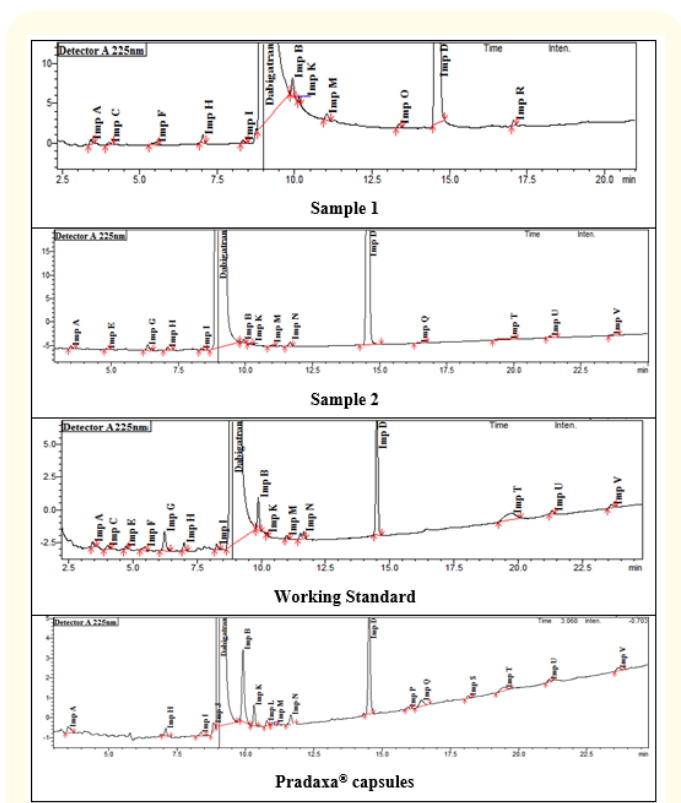


Figure 5: Chromatograms of Dabigatran etexilate and its impurities in samples of API from different sources, in one working standard and in Pradaxa® capsules.

ESI-MS results

The identification of Dabigatran etexilate and its known impurities A,B and C was confirmed by ESI-MS as shown in (Figure 6) and Table 2.

The identification of Dabigatran etexilate and its impurities in API from different sources, in working standard and in Pradaxa® capsules was confirmed by ESI-MS using scan mode from 50-900 mu to produce spectra of molecular weight as shown in Table 3.

Peak detected	Peak name	Retention time RT (min)	Sample 1	Sample 2	Working standard	Pradaxa® capsules
			Area%	Area%	Area%	Area%
1	Imp A	3.423	0.010	0.012	0.005	0.044
2	Imp C	4.013	0.007	-	0.005	-
3	Imp E	4.712	-	0.002	0.003	-
4	Imp F	5.514	0.007	-	0.005	-
5	Imp G	6.234	-	0.028	0.024	-
6	Imp H	7.036	0.016	0.011	0.007	0.028
7	Imp I	8.335	0.007	0.005	0.005	0.020
8	Imp J	8.676	-	-	-	0.029
9	Dabigatran etexilate	8.984	94.515	97.039	99.713	98.707
10	Imp B	9.935	0.033	0.007	0.029	0.340
11	Imp K	10.138	0.008	0.001	0.001	0.076
12	Imp L	10.786	-	-	-	0.020
13	Imp M	11.037	0.014	0.005	0.003	0.010
14	Imp N	11.668	-	0.016	0.012	0.050
15	Imp O	13.356	0.008	-	-	-
16	Imp D	14.619	5.366	2.838	0.142	0.508
17	Imp P	15.959	-	-	-	0.013
18	Imp Q	16.453	-	0.006	-	0.058
19	Imp R	17.076	0.011	-	-	-
20	Imp S	18.157	-	-	-	0.013
21	Imp T	19.494	-	0.016	0.034	0.044
22	Imp U	21.121	-	0.008	0.004	0.014
23	Imp V	23.650	-	0.004	0.005	0.026

- : Not detected in sample or peak is too small to be extracted

Table 1: Impurities of Dabigatran etexilate in the samples and Pradaxa® capsules.

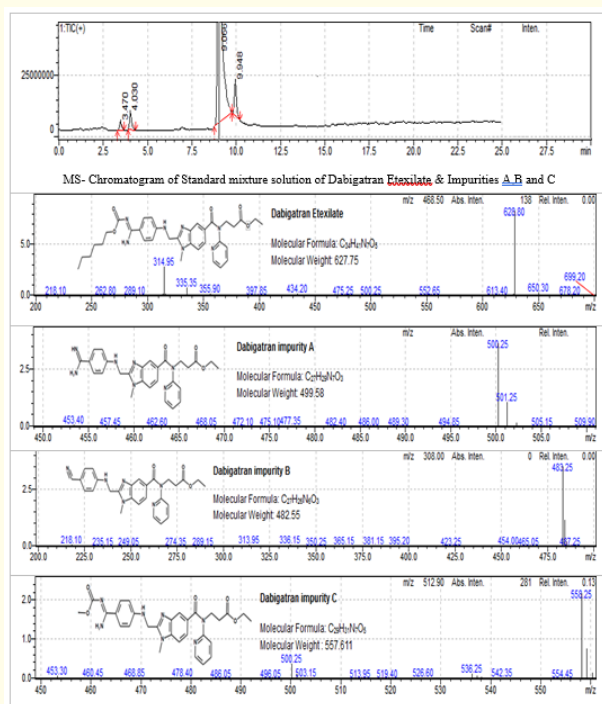


Figure 6: ESI-MS spectra of the protonated ion of Dabigatran etexilate and its known Impurities.

Compound	Retention time (min)	[M+H] ⁺ m/z	Accurate masses m/z
Dabigatran etexilate	8.984	628.80	627.75
Imp A	3.423	500.25	499.58
Imp B	9.935	483.25	482.55
Imp C	4.013	558.25	557.611

Table 2: [M+H]⁺+m/z of Dabigatran etexilate and its known impurities A-E.

Method validation results

Method compatibility with the requirements of system suitability according to the standards of USP 35 was performed, and also based on the requirements of ICH guidelines for validation of analytical procedures.

System suitability: The results of system suitability criterion are described in Table 4.

The calibration curve linearity was examined by studying the correlation coefficient between the concentrations and the response area of each concentration over the calibration ranges tested, it was greater than 0.999 for Dabigatran Etexilate and Impuri-

ties A,B and C. Accuracy was assessed by the recovery percentage. Relative standard deviations for selectivity, repeatability, intermediate precision and robustness were less than 2%. Table 5 Summarizes the results of the method validation tests.

Peak detected	Peak name	Retention time RT (min)	[M+H] ⁺ m/z
1	Imp A (known)	3.423	500.25
2	Imp C (known)	4.013	558.25
3	Imp E (unknown)	4.712	572.25
4	Imp F (unknown)	5.514	501.15
5	Imp G (unknown)	6.234	599.25
6	Imp H (unknown)	7.036	600.25
7	Imp I (unknown)	8.335	614.30
8	Imp J (unknown)	8.676	504.30
9	Dabigatran etexilate	8.984	628.30
10	Imp B (known)	9.935	483.20
11	Imp K (unknown)	10.138	642.30
12	Imp L (unknown)	10.786	656.35
13	Imp M (unknown)	11.037	505.25

14	Imp N (unknown)	11.668	601.25
15	Imp O (unknown)	13.356	684.30
16	Imp D (unknown)	14.619	629.25
17	Imp P (unknown)	15.959	777.30
18	Imp Q (unknown)	16.453	402.40
19	Imp R (unknown)	17.076	643.25
20	Imp S (unknown)	18.157	678.30
21	Imp T (unknown)	19.494	329.25
22	Imp U (unknown)	21.121	643.20
23	Imp V (unknown)	23.650	468.35

Table 3: [M+H]⁺m/z of Dabigatran etexilate and its impurities in the API samples and Pradaxa[®] capsules.

Compound name	RT (min)	Resolution	Tailing factor	Theoretical plates (N)
Impurity A	3.423	-	1.369	5299
Impurity C	4.013	3.308	1.179	9690
Dabigatran	8.984	24.967	1.115	22909
Impurity B	9.935	4.919	1.027	79098

Table 4: The results of system suitability.

Parameters			Dabigatran Etexilate	Imp A	Imp B	Imp C
Linearity	Correlation Factor		0.9998	0.9989	0.9989	0.9996
	Slop		1.827349	2.143666	2.079656	5.250036
Accuracy	50%	R (%)	102.60%	97.30%	98.99%	96.55%
	100%	R (%)	103.25%	101.30%	102.70%	101.76%
Accuracy	150%	R (%)	99.40%	96.60%	101.80%	98.50%
Precision	Repeatability	R (%)	103.90%	100.88%	99.15%	102.56%
		RSD	2.11	2.70	2.85	2.31
Precision	Intermediate Precision	R (%)	99.80%	100.15%	101.89%	98.99%
		RSD	1.99	2.30	2.66	2.15
Robustness	Flow Rate ml/min	0.2	R (%)	105.88%	102.43%	106.12%
			RT	10.426	4.866	11.758
	0.3	R (%)	101.65%	98.95%	103.69%	99.95%
		RT	8.987	3.435	9.925	4.031
0.4	R (%)	97.15%	93.40%	96.52%	94.38%	
	RT	7.452	1.953	8.397	2.612	
Limit of detection (LOD) (µg/ml)			0.002	0.015	0.001	0.012
Limit of quantification (LOQ) (µg/ml)			0.006	0.051	0.003	0.035

Table 5: The results of the method validation tests.

RSD: Relative standard deviation, R: Recovery, RT: Retention time (min)

Discussion

In this method the best separation of Impurities was achieved and the analysis were carried out on an ODS II column (100 x 3.0 mm, 2.2µm particle size), with a mobile phase containing mixture of acetonitrile and water with 0.1% of formic acid in gradient program finalized as (Time/Acetonitrile [0.01/15, 17/60 and 25/80]). The flow rate was 0.3ml/min and UV detector wavelength was set at 225 nm. The retention time was 8.984 min for Dabigatran Etexilate and 3.423 min, 9.935 min and 4.013 min for Impurities A, B, and C respectively, the resolution was >1.5, and it gave acceptable system suitability parameter.

Linearity results show a good correlation between the peak area and concentration with $r \geq 0.9998$ for Dabigatran Etexilate and $r \geq 0.9989$, 0.9989, 0.9996, for Impurities A, B and C respectively, and the calibration curves were linear over the concentration range for Dabigatran Etexilate and its impurities. For the precision and the intermediate precision the RSD values indicated a good method precision, and the accuracy data which were expressed in terms of percentage recoveries were satisfying the acceptance criteria for the study. And for the robustness, the influence of the flow rate was within a specified tolerance range, this shows that method was reproducible and robust, the tailing factors of Dabigatran

Etexilate and its impurities were less than 1.5 and the resolutions were greater than 2.0 in all the deliberate varied chromatographic conditions indicating the robustness of the method. The detection and quantification limits values indicate a good sensitivity of the method.

All the analysis results were acceptable according to the pharmaceutical requirements.

For the analysis of API and pharmaceutical dosage forms

Twenty-two impurities were characterized in all samples, working standards and Pradaxa®, three of them were known impurities (Imp A, B, and C) and the other nineteen were unknown, their $[M+H]^+m/z$ were shown in Table 3, in accordance with USP38-NF33 and ICH guidelines [19,21] none of them (except Imp D) exceeded 0.10% identification threshold, so it is not necessary to characterize the structure of the impurities or identify them, and all analytical results are acceptable according to the pharmaceutical requirements. But Imp D which has 629.25 $[M+H]^+m/z$ exceeded 0.10% the identification threshold, so it was necessary to isolate it by using Semipreparative HPLC and characterize its structure according to MS, NMR H1,C13 and FTIR data which is presented in (Figure 7).

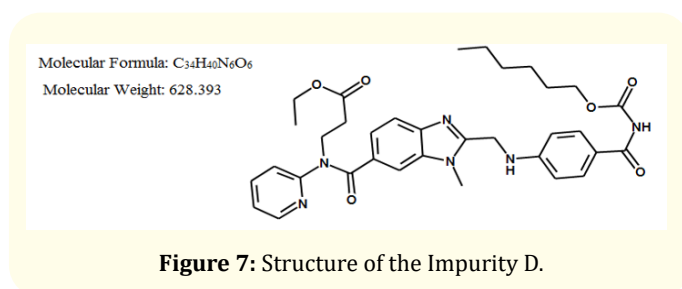


Figure 7: Structure of the Impurity D.

Conclusion

A linear, accurate and precise LC-MS method was successfully developed and validated as per ICH guidelines and according to USP 35 guideline recommendations. The method can be successfully applied for the separation of related substances from Dabigatran Etexilate in the purchased API and for determination of Dabigatran Etexilate in its capsules dosage forms.

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