ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 10, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DABIGATRAN ETEXILATE RELATED SUBSTANCE IN PHARMACEUTICAL DOSAGE FORM BY REVERSE-PHASE – HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Received: 02 May 2018, Revised and Accepted: 22 June 2018

ABSTRACTS

Objective: The objective of the study was to develop and validate new, simple, and selective reverse-phase–high-performance liquid chromatography (RP-HPLC) method for the quantitative determination of Dabigatran Etexilate (DE) and its impurities in pharmaceutical dosage form as per the International Conference on Harmonization guidelines.

Method: Chromatographic analysis was performed on Princeton SPHER-l00 C18 (250 × 4.6 mm, 5 µm) HPLC column, maintained at 50°C column temperatures, 6°C sample tray temperature, and detection monitored at 225 nm. The mobile phase consisted of acetonitrile:phosphate buffer (pH 2.5) (33:67 V/V). The flow rate was maintained at 1.0 ml/min.

Results: The system suitability results indicate good performance of the system. Specificity study indicates that there is no interference of placebo and blank. The percentage relative standard deviation (RSD) of six preparations for known and unknown impurity in the sample solution is found below 10%; hence, the method is precise. The calibration curve for DE (unknown impurity), Impurity A was linear from 0.38 to 4.5 µg/ml (correlation coefficients [r2] for unknown Impurity [DE] and Impurity A are 0.996 and 0.999, respectively). The calibration curve for Impurity B and Impurity E was linear from 0.38 to 9.00 µg/ml (r2 for Impurity B and Impurity E are 0.999 and 0.999, respectively); hence, the method is linear. Accuracy for DE (unknown Impurity), Impurity A, Impurity B, and Impurity E are found within 80%–120%; hence, the method is accurate. The percentage RSD for a standard solution is found below 5% up to 24 h, and percentage impurity change in the sample solution is found below 0.1% up to 18 h; hence, standard solution is stable up to 24 h, and sample solution is stable up to 18 h.

Conclusion: The developed method is new, simple, adequate, specific, precise, linear, and accurate for the determination of DE and its impurities in pharmaceutical dosage forms.

Keywords: Dabigatran etexilate, International Conference on Harmonization Guidelines, Reverse-phase–high-performance liquid chromatography, Method development, Method validations.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i10.27043

INTRODUCTION

Dabigatran Etexilate (DE) (BIBR 1048) is a newly approved oral direct thrombin inhibitor which is indicated for anticoagulation therapy to reduce the risk of strokes and systemic embolism in patients with non-valvular atrial fibrillation. DE is marketed as "PRADAXA" in the form of DE Mesylate (DEM) (BIBR 1048 MS) salt as 75 mg, 110 mg, and 150 mg immediate release capsule. DE is ester prodrug, after oral administration, DE is rapidly absorbed and completely hydrolyzed to its active moiety, Dabigatran (BIBR 953), by non-specific abundant esterases in the gut, plasma, and liver. It is being studied for various clinical indications and in some cases it offers an alternative and beneficial as compare to warfarin as the preferred orally administered anticoagulant ("blood thinner") because it does not require frequent monitoring of the clotting tendency of blood while offering similar results in terms of efficacy [1-4].

Chemically, DEM is a mesylate salt of a prodrug DE of which Dabigatran is an active therapeutic ingredient. DEM contains two ester functional groups (ethyl ester and etexilate ester). The di-ester is essentially a prodrug for the corresponding zwitterion, and the nomenclature and strength are based on the relevant di-ester, intrinsic neutral form [5].

The chemical name for DEM, a direct thrombin inhibitor, is β-Alanine, N-[[2-[[[4-[[[(hexyloxy) carbonyl] amino] iminomethyl] phenyl] amino] methyl] -1-methyl-1H-benzimidazol-5-yl] carbonyl]-N-2-pyridinyl-ethyl ester, methanesulfonate. The empirical formula is C34H41N7O5·CH4O3S and the molecular weight is 723.86 (mesylate salt), 627.75 (DE - free base) and 471.51 (Dabigatran - active moiety), respectively. The structural formulae of DEM, DE, and Dabigatran are presented in Figs. 1-3.

There are several processes and degradation impurities of DE, which are originated through the synthesis process and degradation during storage stability. Even several process impurities and degradation impurities of DE reported in prior literature; there are only three main degradation impurities of DE. The structure and details of the three main impurities A, B, and E are represented in Table 1.

As per literature survey, different assay methods such as UV and HPLC were developed for estimation of Dabigatran from finished dosage form and bulk API [6,7]. There is no HPLC method specified for determination of DE and its related substances in official pharmacopeias (USP and European Pharmacopoeia). However, few methods have been reported in literature for the determination of DE, and its impurities in formulated products which are represented in Table 2.

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 [13]. All these advantages of RP-HPLC make this the first choice of modern chemists. Hence, in this scenario, a reproducible and accurate method of analysis with properly documented validation gives huge support to the pharmaceutical industry. Hence, there is a need to develop newer stability indicating method by HPLC to make it simple and economical. All the above methods are gradient type RP-HPLC methods. Hence, it is needed to develop novel, simple, isocratic, economic HPLC method for separation of DE and impurities. Hence, we proceeded with HPLC method development and validation as per International Conference on Harmonization (ICH) guidelines. The present analytical work comprises simple, precise, rapid, sensitive, and accurate method for the estimation of DE and its known main impurities A, B, and E. Therefore, the present work is aimed to develop a new and economic method for determination of DE in the pharmaceutical formulation in the presence of degradation product. Chromatographic

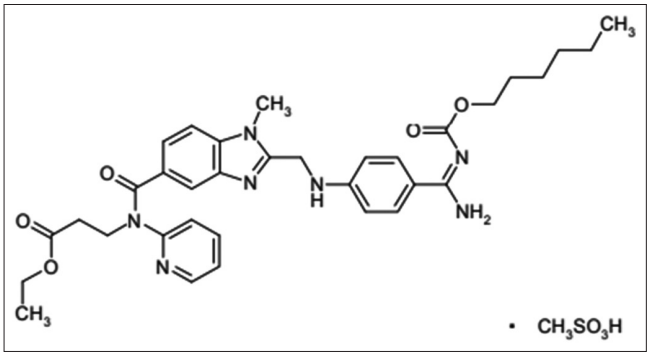


Fig. 1: Dabigatran etexilate mesylate (salt)

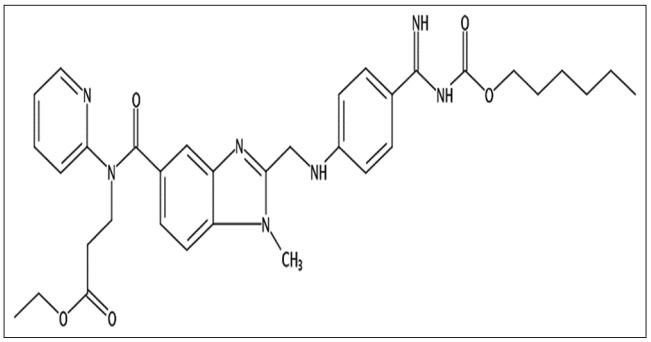


Fig. 2: Dabigatran etexilate (prodrug)

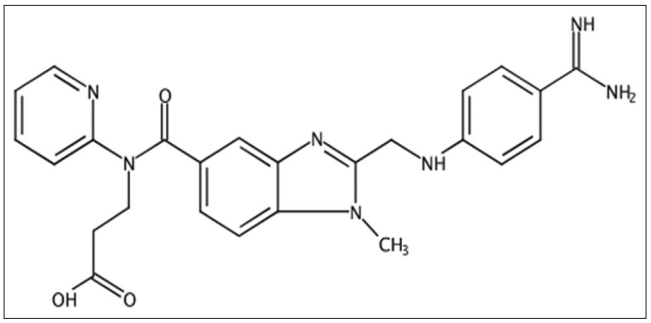


Fig. 3: Dabigatran (active drug)

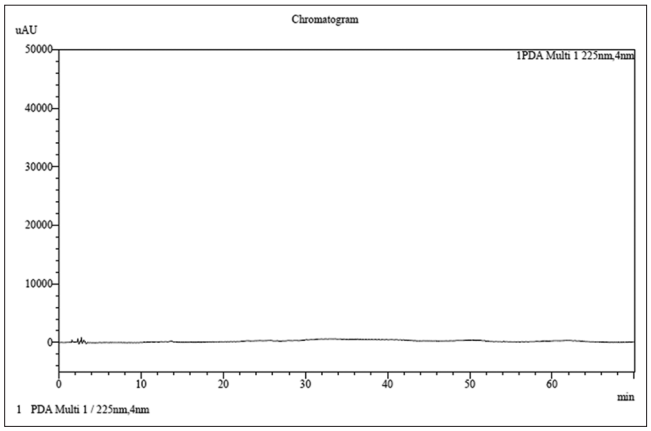


Fig. 4: Chromatogram of blank

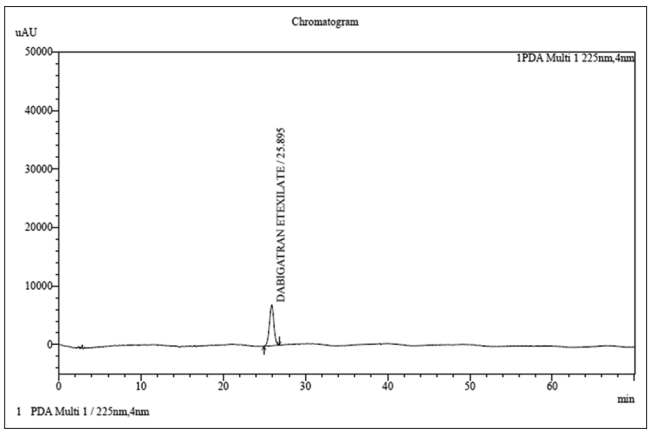
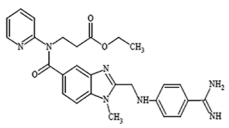
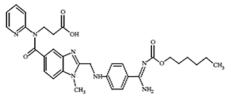
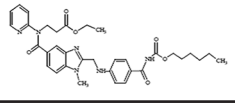


Fig. 5: Chromatogram of Dabigatran etexilate standard

Table 1: The structure and details of three known impurities A, B, and E

Name of the impurity	Structure	Type of impurity
Impurity A: Ethyl-3-(2-(((4-Carbamimidoyl phenyl) amino) methyl)-l-methyl-N-(pyridin-2-yl)-1H-benzo (d) imidazole-5-carboxamido) propanoate		Process related and degradation
Impurity B: 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 and degradation
Impurity E: Ethyl 3-(2-(((4-(((hexyloxy) carbonyl) carbamoyl) phenyl) amino) methyl)-1-methyl N-(pyridin-2-yl) -1 H -benzo [d] imidazole-5-carboxamido) propanoate		Process related and degradation

conditions that give the best resolution with minimal elution time for the DE and its degradation product. This makes the method to be applied in routine work and quantitative determination of the drug and its degradation product. Moreover, it is more sensitive, accurate, and precise method.

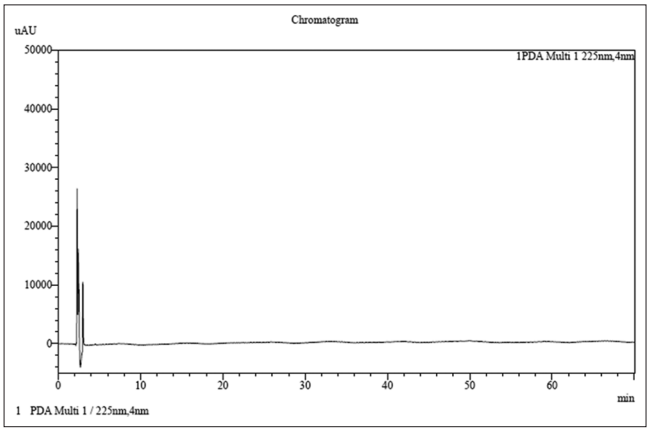


Fig. 6: Chromatogram of placebo

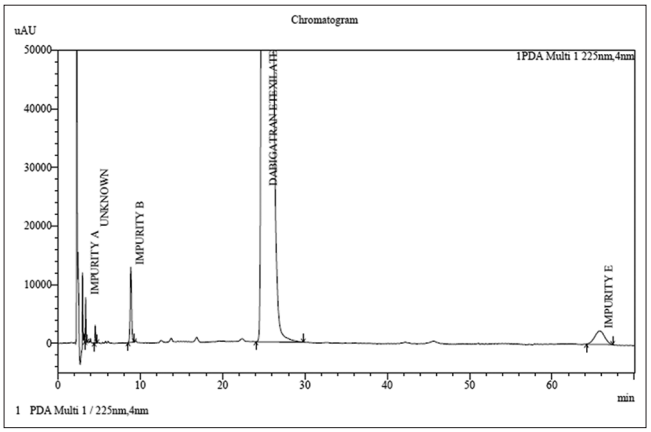


Fig. 7: Chromatogram of Dabigatran Etexilate sample

METHODS

Chemicals and solvents

DEM (drug) and impurities A, B, and E were provided by ZIM Laboratories Limited. All the chemicals and reagents were used in HPLC grade. Potassium dihydrogen phosphate (AR grade) was used for preparing buffer solution and adjusting the pH to 2.5 with 10%

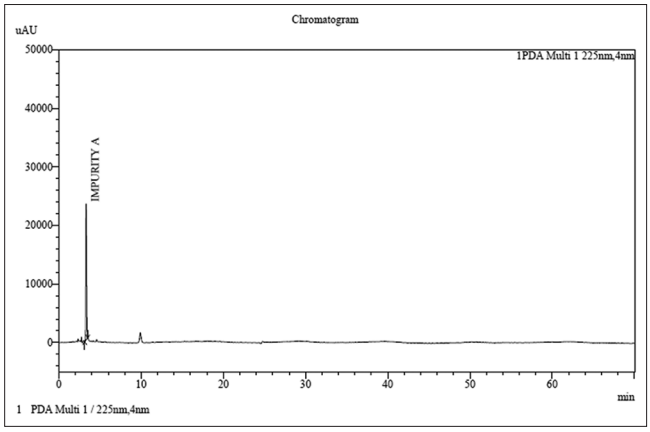


Fig. 8: Chromatogram of Impurity A

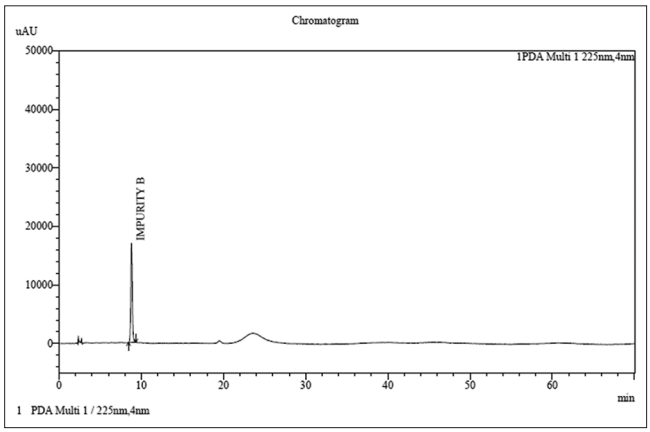


Fig. 9: Chromatogram of Impurity B

Table 2: Literature review on HPLC methods for determination of DE and its impurities [8-12]

Author and y	Eluent mode	Column	Mobile phase	Column temperature (°C)	RT (Min)	Wavelength
Sandeep *et al.*	Gradient	YMC Pack ODS A, 150*4.6 mm, 5mcg	Mobile phase A: Buffer (potassium di hydrogen phosphate) (pH 4.5) mobile phase B: Acetonitrile	--	21.2	220 nm
Dare *et al.*	Gradient	Poroshell 120 EC - 18 (150 mm×4.6 mm, 2.7µ)	Methanol: Buffer (hexane-1 sulfonic acid sodium salt monohydrate (pH 6.5)	30°C	26.97	230 nm
Nagadeep *et al.*	Gradient	Inertsil ODS-3V, 250 mm 4.6 mm, 5 µm	Mobile phase A: Ammonium formate with 0.1% of triethylamine (pH 5) mobile phase B: Acetonitrile	30°C	36.37	255 nm
Sreenivas *et al.*	Gradient	Inertsil ODS-4, 5 m (250 mm×4.6 mm)	Mobile phase A: Phosphate (potassium dihydrogen orthophosphate) buffer (pH 3.0) mobile phase B: Acetonitrile	25°C	24	220 nm
Ravi Kumar *et al.*	Gradient	Inertsil ODS-3 V, 150 mm×4.6 mm, 5 µm	Mobile phase A: Ammonium formate buffer, (pH 4.7) mobile phase B: Acetonitrile	35°C	10.17	220 nm

RP-HPLC: Reverse-phase–high-performance liquid chromatography

Table 3: Optimized chromatographic conditions of DE parameters

Parameter	Solvents
Mobile phase	Acetonitrile:phosphate buffer (pH 2.5) (33:67 v/v)
Column	C18
Diluents	Mobile phase (acetonitrile:phosphate buffer (pH 2.5) (33:67 v/v)
Column temperature	50°C
Wavelength	225 nm
Injection volume	10 µL
Flow rate	1.0 ml/min
Runtime	70
Retention time	25.895

V/V: Volume/volume

Table 4: System suitability results

S. No	RT	Area	Tailing factor	TP
1	26.662	265006	1.063	13174
2	26.632	261320	1.052	13891
3	26.554	265518	1.077	13453
4	26.576	263571	1.063	13718
5	26.52	264489	1.077	13626
Mean±SD	26.5888±0.05773	263981±1652.28		
%RSD	0.21714	0.62591		
Limit	NMT 2%	NMT 5%		

SD: Standard deviation, %RSD: Percent relative standard deviation

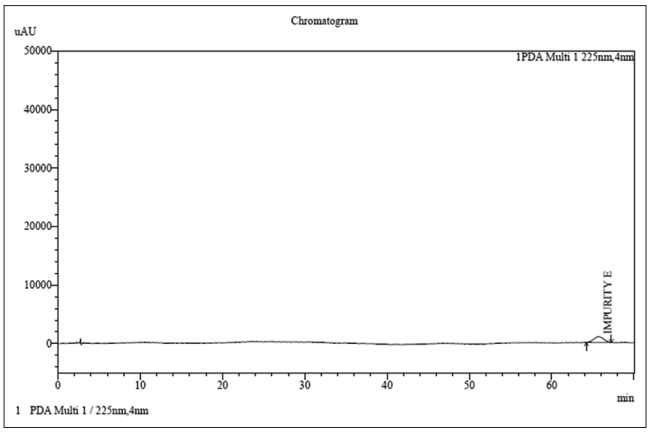


Fig. 10: Chromatogram of Impurity E

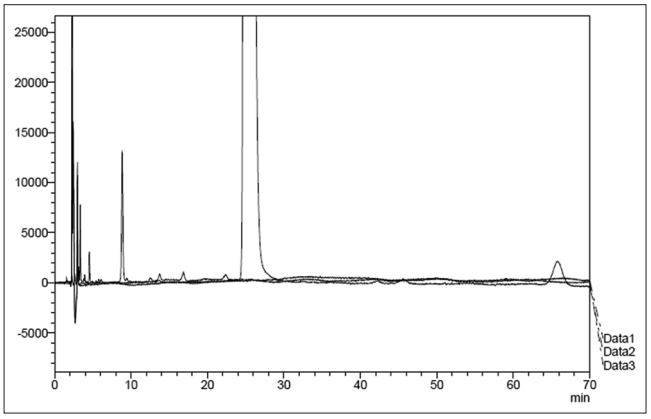


Fig. 11: Overlay chromatograms of blank, placebo, and Dabigatran Etexilate sample

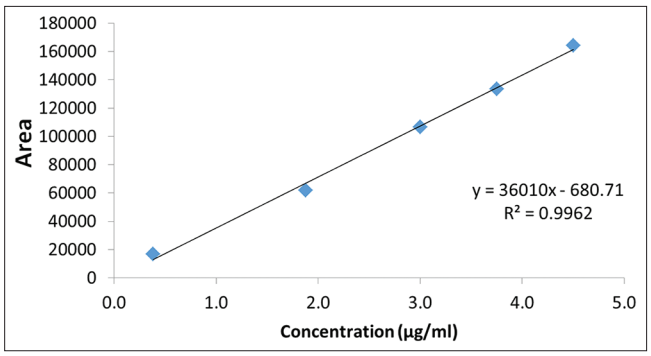


Fig. 12: Linearity graph of unknown impurity (Dabigatran Etexilate)

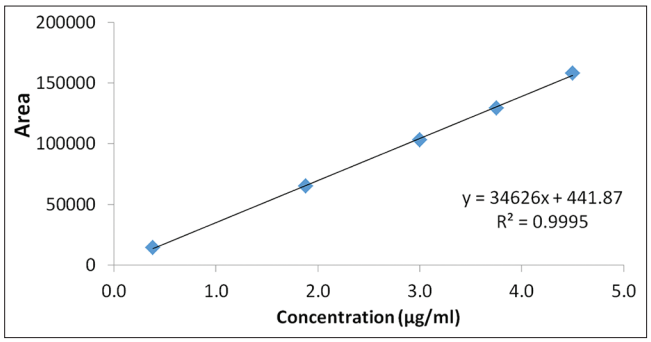


Fig. 13: Linearity graph of Impurity Area

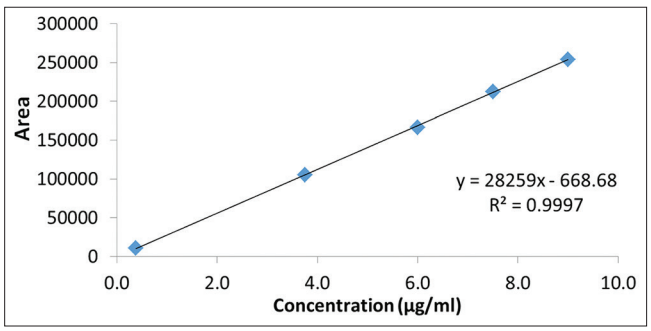


Fig. 14: Linearity graph of Impurity B

phosphoric acid (AR grade). HPLC grade acetonitrile was used for mobile phase preparation.

Instrumental and analytical conditions

Chromatographic analysis was performed on Princeton SPHER-l00 C18 (250 × 4.6 mm, 5 µm) HPLC column, maintained at 50°C column temperatures, 6°C sample tray temperature, and detection monitored at 225 nm. The mobile phase used in this analysis consists of an Acetonitrile:phosphate buffer (pH 2.5) (33:67 V/V). The mobile phase was filtered, degassed before use. The flow rate was maintained at 1.0 ml/min. The injector volume of standard and sample was 10 µL. The solution was injected, and chromatograms were recorded [14]. The optimized chromatographic conditions of DE are in Table 3.

Preparation of mobile phase

Preparation of 0.05 M potassium dihydrogen phosphate buffer pH 2.5 for mobile phase

Weigh and transfer 6.8 g of potassium dihydrogen phosphate in 1000 ml of volumetric flask, add 800 ml of water and sonicate to dissolve for 15

Table 5: Precision results of Impurity A, B, and E and unknown

S. No	Impurity A (%) NMT 0.5%	Impurity B (%) NMT 1%	Impurity E (%) NMT 1%	Highest unknown individual impurity (%) NMT 0.5%	Total impurity (%) NMT 2.5%
1	0.13	0.58	0.52	0.08	1.10
2	0.12	0.56	0.57	0.08	1.25
3	0.12	0.57	0.53	0.08	1.22
4	0.13	0.57	0.60	0.08	1.30
5	0.13	0.57	0.59	0.07	1.29
6	0.13	0.57	0.60	0.08	1.30
Mean	0.13	0.57	0.57	0.08	**1.24**
SD	0.0052	0.0063	0.0354	0.0041	0.0771
RSD	4.0	1.1	6.2	5.1	**6.2**
Limit	NMT 10%	NMT 10%	NMT 10%	NMT 10%	NMT 10%

SD: Standard deviation, %RSD: Percent relative standard deviation

Table 6: Linearity of unknown Impurity (DE)

Sample name	Concentration (µg/ml)	Mean area
LOQ	0.38	16754
50%	1.88	61773
80%	3.00	106535
100%	3.75	133697
120%	4.50	164328
Correlation coefficient (r^2)	0.9962	

DE: Dabigatran Etexilate, LOQ: Limit of quantitation

Table 7: Linearity of Impurity A

Sample name	Concentration (µg/ml)	Mean area
LOQ	0.38	14488
50%	1.88	65094
80%	3.00	103065
100%	3.75	129482
120%	4.50	157884
Correlation coefficient (r^2)	0.9995	

LOQ: Limit of quantitation

Table 8: Linearity of Impurity B

Sample name	Concentration (µg/ml)	Mean area
LOQ	0.38	10811
50%	3.75	105075
80%	6.00	166320
100%	7.50	212931
120%	9.00	254049
Correlation coefficient (r^2)	0.9997	

LOQ: Limit of quantitation

Table 9: Linearity of Impurity E

Sample name	Concentration (µg/ml)	Mean area
LOQ	0.38	9433
50%	3.75	88396
80%	6.00	148065
100%	7.50	182737
120%	9.00	214620
Correlation coefficient (r^2)	0.9991	

LOQ: Limit of quantitation

min, and dilute to volume with water. Adjust pH 2.5 ± 0.05 using dilute (10%) phosphoric acid. Filter the solution using a 0.45 µ filter.

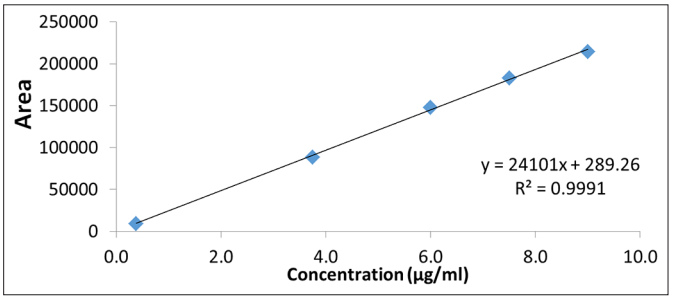


Fig. 15: Linearity graph of Impurity E

Mobile phase

Prepare a filtered and degassed mixture of acetonitrile and buffer pH 2.5 in the ratio of (33:67 v/v).

Preparation of standard solution

Weigh accurately and transfer DEM working standard (about 87 mg) about eq. to 75 mg of DE in 50 ml of volumetric flask, add 25 ml of methanol, sonicate to dissolve for 15 min, and dilute to volume with methanol. Pipette out 5.0 ml of the resulting solution in 50 ml of volumetric flask and dilute to volume with mobile phase. Further, pipette out 5.0 ml of the resulting solution in 100 ml of volumetric flask and dilute to volume with mobile phase (7.5 µg/ml).

Preparation of sample solution

Weigh and remove the content of 20 capsules, weigh accurately and transfer pellets eq. to 300 mg of DE in 200 ml of volumetric flask, add 50 ml of water, sonicate to dissolve for 10 min, stir the sample solution for 30 min by magnetic stirrer, and add about 100 ml of methanol, further stir the sample solution for 10 min by magnetic stirrer, and dilute to volume with methanol. Centrifuge the resultant solution at 2000 rpm for 3 min (if required). Pipette out 5.0 ml of the resulting solution in 10 ml of a volumetric flask and dilute to volume with mobile phase, filter through a 0.45 µ pore size nylon membrane filter (750 µg/ml).

RESULTS AND DISCUSSION

Method validation

This method was validated according to ICH guidelines to establish the performance characteristics of a method to meet the requirements for the intended application of the method. They were tested using the optimized chromatographic conditions and instruments.

System suitability

System performance parameters of HPLC method were determined by injecting 5 replicate injections of standard solutions. Parameters such as retention time, area, tailing factor, and number of theoretical plates (N) were determined. From system suitability studies, it is observed that percentage relative standard deviation (RSD) values for retention

Table 10: Accuracy results for unknown Impurity (DE)

Recovery level	Area	Spiked conc. (µg/ml)	Recovered conc.(µg/ml)	Recovered (%)	Mean recovered (%)
LOQ - 1	14622	0.38	0.410	107.9	107.0
LOQ - 2	13787	0.38	0.390	102.6	
LOQ - 3	15107	0.38	0.420	110.5	
50% - 1	66543	1.91	1.86	97.4	95.1
50% - 2	64616	1.91	1.80	94.2	
50% - 3	64037	1.91	1.79	93.7	
100% - 1	137002	3.82	3.83	100.3	102.4
100% - 2	142491	3.82	3.98	104.2	
100% - 3	140394	3.82	3.92	102.6	
120% - 1	177485	4.59	4.96	108.1	106.9
120% - 2	181911	4.59	5.08	110.7	
120% - 3	167722	4.59	4.68	102.0	

DE: DE: Dabigatran Etexilate, LOQ: Limit of quantitation

Table 11: Accuracy results for Impurity A

Recovery level	Area	Spiked conc. (µg/ml)	Recovered conc.(µg/ml)	Recovered (%)	Mean recovered (%)
LOQ - 1	12524	0.35	0.350	100.0	98.1
LOQ - 2	12466	0.35	0.350	100.0	
LOQ - 3	11761	0.35	0.330	94.3	
50% - 1	63777	1.75	1.79	102.3	102.1
50% - 2	63636	1.75	1.78	101.7	
50% - 3	63931	1.75	1.79	102.3	
100% - 1	129651	3.51	3.63	103.4	103.3
100% - 2	129646	3.51	3.63	103.4	
100% - 3	129457	3.51	3.62	103.1	
120% - 1	158246	4.21	4.43	105.2	106.9
120% - 2	168050	4.21	4.70	111.6	
120% - 3	156337	4.21	4.38	104.0	

LOQ: Limit of quantitation

Table 12: Accuracy results for Impurity B

Recovery level	Area	Spiked conc. (µg/ml)	Recovered conc. (µg/ml)	Recovered (%)	Mean recovered (%)
LOQ - 1	11110	0.34	0.310	91.2	92.2
LOQ - 2	10870	0.34	0.300	88.2	
LOQ - 3	11741	0.34	0.330	97.1	
50% - 1	106776	3.37	2.99	88.7	89.2
50% - 2	107522	3.37	3.01	89.3	
50% - 3	107865	3.37	3.02	89.6	
100% - 1	216495	6.75	6.06	89.8	90.7
100% - 2	220683	6.75	6.18	91.6	
100% - 3	218654	6.75	6.12	90.7	
120% - 1	257831	8.10	7.22	89.1	91.1
120% - 2	272410	8.10	7.62	94.1	
120% - 3	260497	8.10	7.29	90.0	

LOQ: Limit of quantitation

time and area are found within the limit, i.e., not more than 2% and not more than 5%, respectively, which indicates good performance of the system. System suitability results are tabulated in Table 4.

Specificity

It is the ability to asses explicitly the analyte in the presence of components that may be expected to be present. The blank, placebo, standard, sample, Impurity A, Impurity B, and Impurity E solution were prepared and injected in HPLC system for evaluation of specificity of the analytical method.

Observation

The blank and placebo did not show any interference on the retention time of DE, Impurity A, Impurity B, and Impurity E in sample chromatograms. Hence, it is concluded that analytical method is specific for DE, Impurity A, Impurity B, and Impurity E. Typical chromatogram of blank, standard, placebo, sample, Impurity A, Impurity B, Impurity E, and overlay chromatogram is shown in Figs. 4-11, respectively. It revealed that the present analytical RP-HPLC method is specific for DE, Impurity A, Impurity B, and Impurity E.

Precision

The precision of the method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Precision studies were conducted by preparing six different preparations of the sample solution, and results are reported in term of RSD. The percentage RSD of six preparations for known and unknown impurity in the sample solution was found below 10%; hence, method is precise. The results are tabulated in Table 5.

Based on the above results, it revealed that the present analytical RP-HPLC method is precise for DE (unknown impurity), Impurity A, Impurity B, and Impurity E.

Table 13: Accuracy results for Impurity E

Recovery level	Area	Spiked conc. (µg/ml)	Recovered conc. (µg/ml)	Recovered (%)	Mean recovered (%)
LOQ - 1	11115	0.34	0.310	91.2	92.2
LOQ - 2	11698	0.34	0.330	97.1	
LOQ - 3	10765	0.34	0.300	88.2	
50% - 1	102981	3.36	2.88	85.7	87.4
50% - 2	111099	3.36	3.11	92.6	
50% - 3	100903	3.36	2.82	83.9	
100% - 1	196981	6.72	5.51	82.0	90.4
100% - 2	229082	6.72	6.41	95.4	
100% - 3	225053	6.72	6.30	93.8	
120% - 1	253444	8.06	7.09	88.0	88.7
120% - 2	254395	8.06	7.12	88.3	
120% - 3	258357	8.06	7.23	89.7	

LOQ: Limit of quantitation

Table 14: DE standard solution stability results

S. No	Details of SPL	Time (h)	DE area	RSD
1	STD SS 01	0	311733	0.07
2	STD SS 02	6	312612	0.69
3	STD SS 03	12	314081	1.11
4	STD SS 04	18	314177	0.99
5	STD SS 05	24	313756	0.97

DE: Dabigatran Etexilate, RSD: Relative standard deviation

Table 15: DE sample solution stability results

S. No	Details of SPL	Time (h)	Impurity A	Impurity B	Impurity E	Total impurity
1	DE SPL 01	0	0.03	0.24	0.28	0.81
2	DE SPL 02	6	0.04	0.24	0.29	0.83
3	DE SPL 03	12	0.04	0.25	0.33	0.88
4	DE SPL 04	18	0.05	0.24	0.35	0.9
5	DE SPL 05	24	0.06	0.25	0.4	0.98

DE: Dabigatran Etexilate

Linearity

A series of solutions was prepared using DE (unknown impurity), Impurity A, Impurity B, and Impurity E standard from limit of quantitation (LOQ) to 120% of its impurity limits concentration (i.e., DE 0.5%, Impurity A 0.5%, Impurity B 1%, and Impurity E 1%). The calibration curve for DE (unknown impurity), Impurity A was linear from 0.38 to 4.5 µg/ml (r2 for DE=0.996 and 0.999, respectively). The calibration curve for Impurity B and Impurity E was also linear from 0.38 to 9.00 µg/ml (r2 for DE=0.999 and 0.999, respectively). Linearity results of DE (unknown impurity), Impurity A, Impurity B, and Impurity E are tabulated in Tables 6-9, respectively. Linearity graph of unknown Impurity, Impurity A, Impurity B, and Impurity E is represented in Figs. 12-15, respectively.

Accuracy

Accuracy solution was prepared in triplicate from LOQ to 120% by spiking DE (unknown Impurity), Impurity A, Impurity B, and Impurity E of its impurity limits concentration (i.e., DE 0.5%, Impurity A 0.5%, Impurity B 1%, and Impurity E 1%). Accuracy is calculated by impurity added verses impurities recover. Recovery for DE (unknown Impurity), Impurity A, Impurity B, and Impurity E is found within 80% to 120%; hence, method is accurate. Accuracy data of unknown Impurity, Impurity A, Impurity B, and Impurity E are presented in Tables 10-13, respectively.

Solution stability for standard and sample solution

Standard and sample solution was prepared as per method and injected at a different interval such as initial, 6 h, 12 h, 18 h, and 24 h. The percentage RSD for standard solution is found below 5%, and percentage impurity change in the sample solution is found below 0.1% up to 18 h; hence, standard solution is stable up to 24 h, and sample solution is stable up to 18 h. Standard preparation and sample preparation solution stability results are presented in Tables 14 and 15, respectively.

CONCLUSION

In the present study, the developed method is new, simple, adequate, specific, precise, linear, and accurate for the determination of DE and its impurities in pharmaceutical dosage forms.

ACKNOWLEDGMENT

The authors wish to thank ZIM Laboratories Limited, Nagpur, India, for providing the DEM drug, impurities, and facilities to perform research work.

AUTHOR'S CONTRIBUTION

Mr. Shankar Pol, Dr. Rajesh Nawale, and Dr. Vishal Rajkondawar conceived of the presented idea. Mr. Shankar Pol performed analytical method development. Dr. Rajesh Nawale and Dr. Prashant Puranik verified the analytical methods. Dr. Anwar Daud encouraged for research. All authors discussed the results and contributed to the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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