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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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 groups (ethyl ester and etexilate ester). The di-ester is essentially a functional ingredient. DEM contains two ester functional groups (ethyl ester and etexilate ester). The di-ester is essentially an active therapeutic ingredient. DEM contains two ester functional groups (ethyl ester and etexilate ester). The di-ester is essentially an active therapeutic ingredient. DEM contains two ester functional groups (ethyl ester and etexilate ester). The di-ester is essentially an active therapeutic ingredient. DEM contains two ester functional groups (ethyl ester and etexilate ester). The di-ester is essentially an active therapeutic ingredient.
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 it 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)](image)

![Fig. 2: Dabigatran etexilate (prodrug)](image)

![Fig. 3: Dabigatran (active drug)](image)

![Fig. 4: Chromatogram of blank](image)

![Fig. 5: Chromatogram of Dabigatran etexilate standard](image)

| Name of the impurity | Structure | Type of impurity |
|----------------------|-----------|------------------|
| Impurity A: Ethyl-3-2-[((4-Carbamimidoyl phenyl) amino) methyl]-1-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)-1H-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.

**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%

| 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 dihydrogen 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) | Mobile phase A: Methanol; Buffer (hexane-1 sulfo nic 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
phosphoric acid (AR grade). HPLC grade acetonitrile was used for mobile phase preparation.

**Instrumental and analytical conditions**

Chromatographic analysis was performed on Princeton SPHER-100 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
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.

**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

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

| S. No | Impurity A (%) | Impurity B (%) | Impurity E (%) | Highest unknown individual impurity (%) | Total impurity (%) |
|-------|----------------|----------------|----------------|----------------------------------------|-------------------|
|       | NMT 0.5%       | NMT 1%         | NMT 1%         | NMT 0.5%                               | 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              |

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 | 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 | 0.9995 |

| Sample name | Concentration (µg/ml) | Mean area |
|-------------|-----------------------|-----------|
| LOQ         | 0.38                  | 10811     |
| 50%         | 3.75                  | 11760     |
| 80%         | 6.00                  | 166320    |
| 100%        | 7.50                  | 212931    |
| 120%        | 9.00                  | 254049    |
| Correlation coefficient | 0.9997 |

| 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 | 0.9991 |

**Fig. 15: Linearity graph of Impurity E**

y = 24101x + 289.26
R² = 0.9991

**Table 8: Linearity of Impurity B**

| Sample name | Concentration (µg/ml) | Mean area |
|-------------|-----------------------|-----------|
| LOQ         | 0.38                  | 10811     |
| 50%         | 3.75                  | 11760     |
| 80%         | 6.00                  | 166320    |
| 100%        | 7.50                  | 212931    |
| 120%        | 9.00                  | 254049    |
| Correlation coefficient | 0.9997 |

**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 | 0.9991 |

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 assess 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.

**Tables**

| 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 Eteixlate, LOQ: Limit of quantitation
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 Table 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 is represented 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.

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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.

REFERENCES

1. Hauel N, Ries U, Priepke H, Wienen W, Stassen JM, Inventor; Boehringer Ingelheim Pharma AG, Assignee. Disubstituted Bicyclic
Heterocycles, their Preparation and their use as Medicaments. WIPO Patent Cooperation Treaty (PCT) Patent Application WO1998037075. 1998. p. 1-201.

2. Stangier J, Rathgen K, Stähle H, Gansser D, Roth W. The pharmacokinetics, pharmacodynamics and tolerability of dabigatran etexilate, a new oral direct thrombin inhibitor, in healthy male subjects. Br J Clin Pharmacol 2007;64:292-303.

3. Boehringer Ingelheim. USFDA PRADAXA (Dabigatran Etxetilate Mesylate) Capsules for Oral use Label/Patient Information Leaflets. US@FDA; 2010. p. 1-9.

4. Drugbank.ca/drugs/DB06695. Dabigatran Etxetilate Mesylate-Drug Bank; c2018. Available from: http://www.drugbank.ca/drugs/DB06695. [Last cited on 2018 Apr 22].

5. PRADAXA (Dabigatran Etxetilate Mesylate) Capsules 75mg and 150mg, NDA 22-512, Center for Drug Evaluation and Research; 2010. p. 1-71.

6. Kumar VD, Balaraju K, Kumar AA. A Rapid RP-HPLC method development and validation for the quantitative estimation of dabigatran etexilate mesylate in capsules. Int J Pharm Pharm Sci 2015;7:352-6.

7. Hussain SS, Bhavani G, Kumar AA. UV spectrophotometric assay method development and validation of dabigatran etexilate in capsules. Int J Pharm Pharm Sci 2015;7:286-9.

8. Bomma S, Haque MA, Reddy PR, Bakshi V. Method development and validation for the determination of related compounds in dabigatran etexilate mesylate by RP-HPLC. Int J Med Nanotechnol 2014;1:186-93.

9. Dare M, Jain R, Pandey A. Method validation for stability indicating method of related substance in active pharmaceutical ingredients dabigatran etexilate mesylate by reverse phase chromatography. J Chromatogr Sep Tech 2015;6:1-10.

10. Nagadeep J, Kamaraj P, Arthanareeswari M. Gradient RP-HPLC method for the determination of potential impurities in dabigatran etexilate in bulk drug and capsule formulations. Arabian J Chem 2015. (Press).

11. Sreenivas N, Raghu BK, Douglas SP, Ray UK. Validation of stability-indicating reverse phase HPLC method for the determination of related substances in dabigatran etexilate mesylate drug substance. Pharm Lett 2015;7:272-9.

12. Hanmini RB, Maram RK, Woo HC, Venkat BR, Pasagadagula V. QBd approach method development for estimation of dabigatran etexilate along with its impurities and identification of degradants in capsule dosage form. Am J Anal Chem 2016;7:494-524.

13. Lee CR, Guivarch F, Van Dau CN, Tessiera D, Krstulovic AM. Determination of polar alkylating agents as thiocyanate/isothiocyanate derivatives by reaction headspace gas chromatography. Analyst 2003;128:857-63.

14. Kulkarni AA, Vaidya IS. Flow injection analysis: An overview. J Crit Rev 2015;2:19-24.