Development and validation of RP-HPLC method for the estimation of dabigatran etexilate mesylate (DEM) in bulk form

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ABSTRACT
The RP- HPLC method was developed and validated for the determination of Dabigatran Etexilate Mesylate (DEM) in bulk form. The chromatography was carried out on Unisol C18 (4.6 × 150 mm, 3μm) using a mobile phase of methanol and ammonium acetate buffer in the ratio of 90:10, at a flow rate of 1 ml/minute. The analytes were monitored at 226 nm using a PDA detector. The retention time of the DEM was 2.52 min. The method was linear in the concentration range of 20-100 µg/ml with a correlation coefficient of 0.999. The method was validated as per ICH guidelines.

Keywords: HPLC, Method Validation, Dabigatran Etexilate Mesylate, ICH guidelines.

INTRODUCTION[1,2]
DEM is chemically β-Alanine, N-[[2-[[4-[[((hexyloxy) carbonyl] amino] phenyl] amino] methyl]-1-methyl-1H-benzimidazol-5-yl]carbonyl]-N-pyridinyl-,ethyl ester,methanesulfonate. DEM is an orally available mesylate salt form of the etexilate prodrug of dabigatan and a direct thrombin inhibitor with anticoagulant activity. Thrombin, a serine protease, is responsible for the conversion of fibrinogen to fibrin in the coagulation cascade. Inhibition of thrombin consequently prevents thrombus development. Dabigatran inhibits free thrombin, fibrin-bound thrombin and thrombin-induced platelet aggregation, which results in a prolongation of a PTT (partial thrombo plastin time), ECT (Ecarin clotting time), and TT (thrombin time). It is used in embolism associated with atrial fibrillation, cardioversion of atrial fibrillation/flutter, thromboprophylaxis in orthopaedic surgery, cerebral embolism, and treatment of acute venous thromboembolism. DEM is available in the form of Capsules with the Brand name of PRADAXA (Marketed by Boehringer Ingelheim, India) with strengths 75 & 150 mg.
The literature survey reveals that various analytical methods like Spectrophotometric and HPLC were reported for the determination of DEM in formulations, but there were no reported HPLC methods in bulk form. Moreover, the available HPLC methods for the determination of dabigatran in formulations were time consuming for elution and uneconomical. Hence an attempt was made to develop, a simple, precise, accurate, robust, and economical RP-HPLC method for the estimation of DEM in bulk.

MATERIALS AND METHODS

Instrumentation

HPLC Agilent 1260 Infinity - PDA detector with auto sampler, Unisol C18 (4.6 ×150mm, 3µm particle size) equipped with Open lab EZ Chrome software, Mettler Toledo ME 204 balance, Eutech pH 700 pH meter.

Chemicals and solvents

DEM was obtained as a gift sample from MSN Laboratories, Hyderabad, India and was used without further purification. All chemicals and reagents used were of HPLC grade. HPLC grade methanol, ammonium acetate and water were procured from Merck Pharmaceuticals Private Ltd., Mumbai, India. Glacial acetic acid was purchased from Qualikems, Vadodara, India.

Preparation of standard stock solution

DEM is accurately weighed (100mg) and dissolved in 100ml of HPLC grade methanol to get 1000 µg/ml stock solution. Working standard solutions were further diluted with diluent (Methanol : Acetate buffer 60:40 v/v) to get concentrations of 20-100µg/ml. Each of these drug solutions (10µl) were injected into the column, the peak area and retention times were recorded.

METHOD DEVELOPMENT

Chromatographic conditions

Isocratic elution was performed using the mobile phase composition of methanol and ammonium acetate buffer (pH 5.0). Initially trails were conducted using mobile phase consisting of methanol and ammonium acetate buffer in the ratio of 80:20. Unisol C18 column (150x4.6mm,3µm), pH of 5.0 at a flow rate of 1 ml/min, further the trials were conducted by changing the mobile phase composition to 85:15 v/v of methanol and ammonium acetate buffer and then the method was optimized by changing the mobile phase composition to 90:10 v/v of methanol and buffer, where acceptable peak characteristics were obtained with a good retention time (2.52 min).
The proposed method was validated for following parameters according to ICH guidelines: System suitability, Linearity, Accuracy, Specificity, Precision, Limit of Detection (LOD) and Limit of Quantitation (LOQ), Robustness.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Obtained value</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tailing Factor</td>
<td>1.07</td>
<td>NMT 2</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>8716</td>
<td>NMT 2000</td>
</tr>
<tr>
<td>%RSD of 6 injections</td>
<td>0.09</td>
<td>NMT 2%</td>
</tr>
</tbody>
</table>

Method validation

Fig. 2: Counter plot of DEM standard-100µg/ml

Fig. 3: Three dimensional view of chromatogram of DEM standard-100µg/ml

Table 1: Results for regression analysis of DEM

<table>
<thead>
<tr>
<th>S. NO</th>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Regression equation (Y)</td>
<td>y=20, 86.083x+13,211,131+06</td>
</tr>
<tr>
<td>2</td>
<td>Correlation coefficient (R)</td>
<td>0.999</td>
</tr>
<tr>
<td>3</td>
<td>Slope (m)</td>
<td>2086083</td>
</tr>
<tr>
<td>4</td>
<td>Y – intercept (c)</td>
<td>1321131</td>
</tr>
<tr>
<td>5</td>
<td>Range</td>
<td>20 – 100µg/mL</td>
</tr>
<tr>
<td>6</td>
<td>Limit of detection (LOD)</td>
<td>1.5 µg/mL</td>
</tr>
<tr>
<td>7</td>
<td>Limit of quantitation (LOQ)</td>
<td>2.5 µg/mL</td>
</tr>
</tbody>
</table>

System suitability test

100 µL of the standard solution was injected under optimized chromatographic conditions to evaluate the suitability of the system. The system suitability test parameters were noted.
Specificity

Chromatograms of standard and blank were recorded and chromatogram of blank did not show any peak at the retention time of analyte. This shows that the method is specific.

![Blank chromatogram of DEM](image1)

**Fig. 4: Blank chromatogram of DEM**

![Standard chromatogram of DEM](image2)

**Fig 5: Standard chromatogram of DEM**

Linearity

The calibration curve was obtained with five concentrations of the standard solution (20–100 μg/ml for HPLC method and 2–10 μg/ml for UV method). The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

![Calibration curve of DEM](image3)

**Fig. 6: Calibration curve of DEM**
Table 3: Linearity results for DEM

<table>
<thead>
<tr>
<th>Concentration(µg/ml)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>43570194</td>
</tr>
<tr>
<td>40</td>
<td>84569316</td>
</tr>
<tr>
<td>60</td>
<td>128580363</td>
</tr>
<tr>
<td>80</td>
<td>169100695</td>
</tr>
<tr>
<td>100</td>
<td>207931105</td>
</tr>
</tbody>
</table>

Correlation coefficient: 0.999
Slope (m): 2086083
Intercept (c): 1321131

Accuracy

The accuracy of the method was evaluated by recovery study of DEM at three concentration levels (50%, 100% and 150%). A study was carried out in triplicate at 2, 4 and 6 µg/ml in UV and 20, 40 and 60 µg/ml in HPLC. A fixed amount of pre-analysed sample and standard drug was added and recovery was studied for the quantification of the DEM. The percentage recovery and mean % recovery were calculated.

Table 4: Accuracy results for DEM

<table>
<thead>
<tr>
<th>S.NO</th>
<th>% Level</th>
<th>Standard amount</th>
<th>Spiked amount</th>
<th>Amount found</th>
<th>% Recovery</th>
<th>Mean % Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 %</td>
<td>40</td>
<td>20</td>
<td>59.81</td>
<td>99.68</td>
<td>Mean=99.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>20</td>
<td>59.65</td>
<td>99.41</td>
<td>SD = 0.5006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>20</td>
<td>59.23</td>
<td>98.71</td>
<td>%RSD=0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>40</td>
<td>78.97</td>
<td>98.71</td>
<td>Mean =99.20</td>
</tr>
<tr>
<td>2</td>
<td>100 %</td>
<td>40</td>
<td>40</td>
<td>79.51</td>
<td>99.38</td>
<td>SD = 0.436</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>40</td>
<td>79.63</td>
<td>99.53</td>
<td>%RSD=0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>60</td>
<td>99.13</td>
<td>99.13</td>
<td>Mean =99.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>60</td>
<td>98.96</td>
<td>98.96</td>
<td>SD =0.087</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>60</td>
<td>99.01</td>
<td>99.01</td>
<td>%RSD=0.09</td>
</tr>
</tbody>
</table>

Precision

The precision was determined for DEM in terms of intraday and interday. For Intraday precision evaluation, standard solution (100 µg/ml for HPLC and 6 µg/ml of UV) was prepared from stock solution and injected into the system six times (n=6) at two different times in a day. The interday precision was studied by injecting the same concentration of standard solutions into the system six times on consecutive days. The standard deviation and the relative standard deviation were reported for precision. RSD for peak areas should be NMT 2%, which indicate the precision of the developed methods.

Table 5: Intraday and Interday precision results for DEM

<table>
<thead>
<tr>
<th>S. No</th>
<th>Intraday peak area</th>
<th>Interday peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>212262708</td>
<td>212262509</td>
</tr>
<tr>
<td>2</td>
<td>212438592</td>
<td>212438523</td>
</tr>
<tr>
<td>3</td>
<td>212047244</td>
<td>212046131</td>
</tr>
<tr>
<td>4</td>
<td>212008206</td>
<td>212007991</td>
</tr>
<tr>
<td>5</td>
<td>211999883</td>
<td>211999853</td>
</tr>
<tr>
<td>6</td>
<td>211926882</td>
<td>209925998</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.09</td>
<td>0.44</td>
</tr>
</tbody>
</table>
Limit of detection and limit of quantitation

The limit of detection and the limit of quantitation of the developed method was studied by injecting progressively low concentrations of the standard solution using the developed HPLC method. The LOD & LOQ values were found as 1.5 and 2.5 μg/ml, which indicates the sensitivity of the method.

Robustness

Robustness of the method were determined by altering slight changes in the chromatographic conditions like change in flow rate, buffer pH and mobile phase composition in HPLC and detection wavelength. It was observed that there were no marked changes in chromatograms and spectrum obtained, which demonstrated that the developed HPLC and UV methods were robust.

<table>
<thead>
<tr>
<th>Table 6: Results of Robustness for DEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>Flow Rate</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Buffer pH</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td>Mobile phase</td>
</tr>
<tr>
<td>composition</td>
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<td></td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

A reverse phase liquid chromatographic method was developed and validated for the determination of DEM in bulk with UV detection at 226 nm by using Unisol C18 column with mobile phase composition methanol and ammonium acetate buffer (adjusted to pH 5.0 with glacial acetic acid) in the ratio of 90:10. The UV absorption counter plot and three dimensional view of chromatogram were shown in Fig 2 and 3. The system suitability, results were shown in table no.2. The specificity of the analytical method was established by injecting the 10μl solutions of diluent, standard individually to investigate interference from the representative chromatograms in Fig 4 and 5. The method was linear in the range of 20-100 μg/ml. The linearity results were shown in table no.3. The calibration curve was shown in Fig 6. The results of recovery studies were shown in table 4. The % RSD for intraday and interday precision were found as 0.09 and 0.44, which indicates the method is precise. The results of precision studies were shown in table 5. The LOD & LOQ values were found as 1.5 and 2.5 μg/ml, which indicates the sensitivity of the method. The robustness results were shown in table no.6.

CONCLUSION

The developed RP-HPLC method was simple, rapid, accurate, precise and sensitive for the quantification of DEM and is LC-MS compatible which can be efficiently used for further LC-MS analysis. The method rely on the use of simple working procedure, and hence this method can be routinely employed in quality control for analysis of DEM from bulk drug.

ACKNOWLEDGEMENT

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