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Development and Validation of Stability-Indicating RP-LC Method for the Determination of Dabigatran Etexilate Mesylate in Bulk and Pharmaceutical Formulations

[1]Asmaa A. El Zaher, [1]Ehab F. El Kady, [2]Ola M. El Houssini and [2]Hind E. El Ghwas

[1]Pharmaceutical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr El-Aini St., Cairo 11562, Egypt.
[2]National Organization for Drug Control and Research (NODCAR), 51 Wezerat El- Zerah Street, Agouza. P.O. Box 12553 Giza, 35521, Egypt.

ABSTRACT

A simple, precise, accurate and rapid stability indicating liquid chromatography (LC) method was developed for the determination of dabigatran etexilate mesylate (DEM) in bulk powder and pharmaceutical formulations. Chromatographic separation was carried out by isocratic elution on Eclipse XDB C_8 (4.6×250 mm, 5 µm) using 0.01 M orthophosphoric acid (pH 2.6): acetonitrile (60:40 v/v) as the mobile phase. The flow rate was 1.5 ml min^{-1} and the UV detection was done at 225 nm. Linearity was obtained over a concentration range of 5-100 µg ml^{-1} with regression coefficient of 0.9975. The values for LOQ and LOD were found to be 0.221µg ml^{-1} and 0.067 µg ml^{-1} respectively. Moreover the method was successfully used for the separation of DEM from its degradation product (dabigatran etexilate o-desethyl). The method was validated as per ICH guidelines.

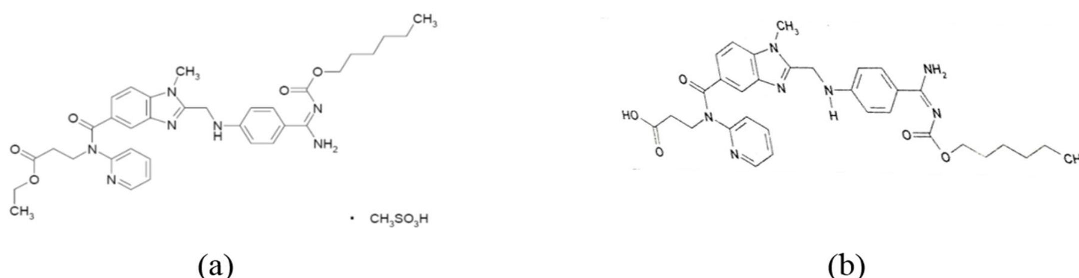
Key words: Dabigatran etexilate mesylate, Pharmaceutical preparation, RP-LC Stability indicating assay, RP-HPLC and method validation.

Introduction

Dabigatran etexilate mesylate (DEM) is β-alanine, N-[[2-[[[4-[[[(hexyloxy) carbonyl] amino] phenyl] amino] methyl]-1-methyl-1H-benzimidazol-5-yl] carbonyl]-N-pyridinylethylester, methane sulfonate (Fig.1a) (Roy *et al*., 2014). DEM is an inactive prodrug that is converted to dabigatran. Dabigatran is the main active principle in plasma, a rapid-acting competitive and reversible direct inhibitor of thrombin (Geetharam *et al*., 2014; Leko, 2014 and Garnock-jones, 2011). Thrombin (serine protease) enables the conversion of fibrinogen into fibrin during the coagulation cascade. Thus its inhibition prevents thrombus development (Sandeep *et al*., 2014), which resulted in prolongation of partial thrombo plastin time (PTT), Ecarin clotting time (ECT) and thrombin time (TT) (Geetharam *et al*., 2014). Dabigatran etexilate mesylate (DEM) is an alternative to Warfarin because it does not required frequent blood tests for international normalized ratio (INR) monitoring, while offering similar results in terms of efficacy (Dare *et al*., 2015) and alternative to vitamin-K-antagonists (Stöllberger and Finsterer, 2012). Also, DEM is indicated in patients with atrial fibrillation. It prevented venous thromboembolism in patients who have undergone total hip or knee replacement surgery (Prajapati *et al*., 2014). Some impurities in DEM such as unreacted starting materials, by-products of the reaction, products of side reactions and degradation products are undesirable, and in extreme cases, might even be harmful to a patient being treated with a dosage form containing the active pharmaceutical ingredient (API). Therefore, there is a need for determination of the level of any impurity in dabigatran samples (Jayaraman *et al*., 2013). There are few analytical methods reported in literatures for analysis of DEM which includes HPLC method (Bernardi *et al*., 2013; Shelke and Chandewar, 2014; Reddy and Rao, 2014; Khan *et al.,* 2014; Damle and Bagwe, 2014 and Bakshi and Singh, 2002), UPLC-MS/MS method in human plasma (Delavennea *et al*., 2012), LC/MS method (Li *et al*., 2014 and Hu *et al*., 2013), GC/MS method (Balaji *et al*., 2012) and Spectrophotometric methods (Roy *et al*., 2014; Dare *et al*., 2015 and Hussain *et al.,* 2015). One of the degradation products of dabigatran is (BIBR 1087) (Paul, 2010) which is qualified up to the specified limit (EMEA, 2008). Therefore, the present work is aimed to develop new and economic method for determination of DEM in bulk powder and in pharmaceutical formulation in presence of degradation product (Dabigatran Etexilate O-Desethyl (DEG) BIBR 1087) (Fig.1b). The DEG is (2-(2-((4-(N''-(hexyloxy carbonyl) carbamimidoyl) phenylamino) methyl)-1-methyl-N-(pyridine-2-yl)-1 H-benzo[d] imidazole-5-carboxamido) acetic acid), it is acid obtained from ester hydrolysis of DEM. The proposed method has the advantage over the other reported methods (Bernardi *et al.,* 2013) in the selection of

Corresponding Author: Hind E. El Ghwas, National Organization for Drug Control and Research (NODCAR), 51 Wezerat El- Zerah Street, Agouza. P.O. Box 12553 Giza 35521, Egypt.
E-mail: honey_elghwas@hotmail.com

chromatographic conditions that give the best resolution with minimal elution time for the DEM 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



(a) (b)

Fig.1: (a) Molecular structure of Dabigatran Etexilate Messylate (DEM) and (b) its degradation product (Dabigatran Etexilate O-Desethyl) (DEG).

Experimental

Instrumentation

An Agilent 1200 HPLC system (Germany), equipped with vacuum degasser, quaternary-pump, column oven and UV/Vis detector was used. Separation and quantitation were made on Eclipse XDB C8 (250mm × 4,6mm, 5µm) – YMC, USA. Elma S100 ultrasonic processor model KBK 4200 (Germany) was used.

Reagents and reference samples

Pharmaceutical grade DEM (certified to contain 99.80%), DEG (certified to contain 98.30%), pradaxa® 75mg tablets nominally containing 86.6 mg of DEM equivalent to 75 mg dabigatran etexilate per tablet and pradaxa® 110 mg tablets nominally containing 126.9 mg of DEM equivalent to 110 mg dabigatran etexilate per tablet were supplied by Boehringer Ingelhieim-Egypt. All solvents used were LC grade and all reagents were of analytical grade. Ethanol, methanol and acetonitrile were purchased from Sigma-Aldrich (Germany). Orthophosphoric acid (85%) was purchased from VWR Chemicals (Pool, England). Bi-distilled water was in-house product by water still BOECO Germany. Membrane filters 0.45µm from Teknokroma (Barcelona, Spain) were used. Orthophosphoric acid 0.01 M was prepared by diluting 0.7 ml of orthophosphoric acid to 1000 ml bi-distilled water (pH 2.6), then the solution was filtered through 0.45µm membrane filter. Two diluents were prepared by mixing acetonitrile: bi-distilled water (50:50 v/v) (diluent 1) and acetonitrile: ethyl alcohol (80:20 v/v) (diluent 2).

Chromatographic conditions

Chromatographic separation was achieved using the previously mentioned instrumentation and applying isocratic elution based on a mobile phase consisting of 0.01M orthophosphoric acid (pH= 2.6): acetonitrile (60:40 v/v*)*. The mobile phase was pumped through the column at a flow rate of 1.5 ml min^{-1}. The Analysis was performed at controlled column temperature 25°C and detection was carried out at 225 nm. The injection volume was 20µl.

Preparation of DEM standard stock solution

Accurately weighted 25 mg of DEM was transferred into a 25 mL volumetric flask, dissolved in and completed to the volume with diluent 1 (1 mg ml^{-1}).

Preparation of DEM working solution

The previously prepared stock solution of DEM (5 ml) were transferred to a 50 ml volumetric flask and the volume was completed to mark with diluent 1 (100 µg ml^{-1}).

Preparation of DEG stock solution

Accurately weighted 10 mg of DEG was transferred into a 10 ml volumetric flask dissolved in and completed to volume with methanol (1 mg ml^{-1}**).**

Sample solution preparation

Twenty pradaxa® 75 mg or Pradaxa® 110 mg were separately opened and the contents of each were grounded then mixed well. Accurately weighted 183 mg and 270 mg of the grounded pellets equivalent to 75 mg and 110 mg of DEM were transferred to 75 ml and 100 ml volumetric flask, respectively and dissolved in as diluent 2. The two solutions were sonicated for 15 min with intermediate shaking. Finally, the volumes were completed to prepare 1 mg ml^{-1} and 1.1 mg $ml^{-1}$for pradaxa 75 mg and pradaxa 110 mg respectively. The solutions were filtered through 0.45 membranes, followed by serial dilution to the required concentrations using (diluent 1).

Procedure

Calibration curves

Different aliquots from DEM standard stock solutions were transferred into series of 10 ml volumetric flasks, the volumes were completed with diluent 1 to give solutions containing 5-100 µg ml^{-1} DEM. 20 µl of each solution was injected in triplicates into the chromatograph. A calibration curve was obtained by plotting area under the peak (AUP) against concentration(C).

Assay of DEM in bulk

The procedure mentioned in section 2.6.1 was repeated using concentrations equivalent to 20- 80 µg ml^{-1} DEM in bulk.

Assay of DEM in pharmaceutical preparations

The sample solutions prepared in section 2.5 were serially diluted using concentrations equivalent to 8-40 µg ml^{-1} and 10-77 µg ml^{-1} for pradaxa® 75 mg and Paradaxa® 110 mg, respectively. The solutions were injected in triplicates (Fig.2). The recovered concentrations of DEM were calculated using calibration equation.

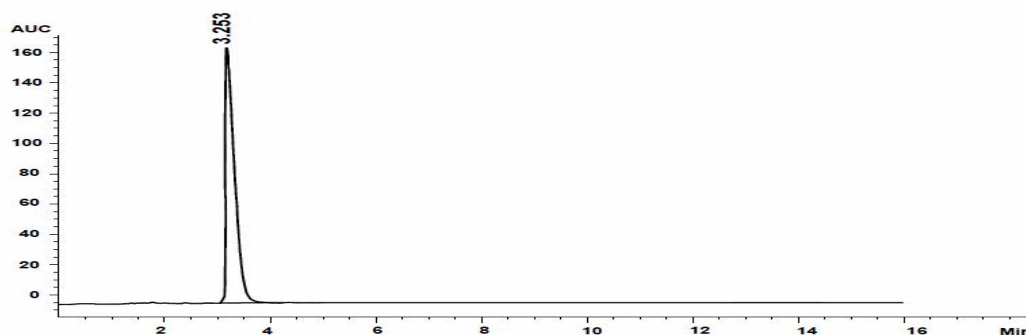


Fig. 2: A typical LC chromatogram of 40µg mL^{-1} of Paradaxa® sample.

Assay of DEM in laboratory prepared mixtures with DEG

The procedure mentioned in section 2.6.1 was repeated using concentrations equivalent to 15-95 µg mL^{-1} and from 1% to 30% of DEG, all dissolved in diulent 1. The mixtures were injected in triplicates (Fig.3). Recovered concentrations of DEM were calculated from the calibration equation.

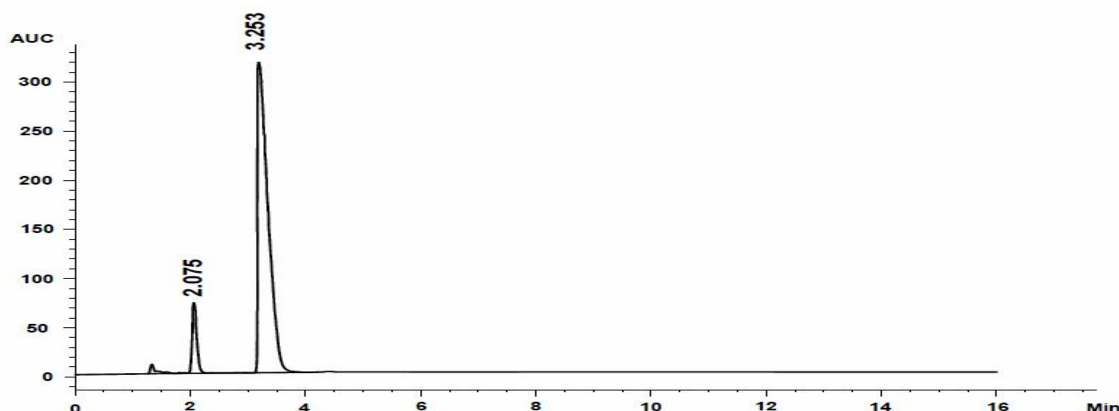


Fig. 3: A typical chromatogram of [1] Dabigatran Etexilate O-Desethyl and [2] DEM in laboratory prepare mixture.

Results and Discussion

Method development

The core intention of this paper was to develop a stability indicating assay for DEM with short run time. Therefore, the conditions affecting the chromatographic performance were carefully studied in order to recognize the most suitable chromatographic system for determination of DEM in presence of its degradation product. The choice was based on the highest number of theoretical plates and the best resolution.

The chromatographic separation was tried using C_{18} columns, including Novoback, Hypersil and ACE with different dimension (150×4.6 mm, 5µm, 250×4.6 mm, 5µm and 250×4.6 mm, 5µm) and the result indicated that, inadequate resolution, broad peak and long retention time were obtained with the previous columns. However, when using Eclipse XDB C8 (250×4.6 mm, 5µm) less hydrophobic column; there was better peak shapes within reasonable run time and satisfactory separation. So we indicate that, the column Eclipse XDB C8 is the best choice for this study. On the other hand, the effect of changing column temperature on chromatographic performance was investigated using column temperature of (25, 35, and 40°C) and it was found that, increasing the temperature poorly shortened retention times for DEM and its degradant, so minor resolution change occur. Therefore, ambient temperature of 25°C ±2 was chosen.

Several mobile phases were evaluated using various proportions of different aqueous phases and organic modifiers. Methanol (as organic modifier) was tried and broad peaks with insufficient separation were obtained. However, when using mobile phases containing (40, 20 and 10 %) of acetonitrile, it was found that, the peak skewing occurred with increasing the ratio of acetonitrile in the mobile phase. While, it had slight decreasing effect on the retention time. According to these results, the mobile phase containing 40 % acetonitrile was enough to give sharp symmetric peaks within a short run time with reasonable resolution and sensitivity. On the other hand, when using mobile phases containing (60, 80 and 90 %) of orthophosporic acid, increased the ratio of orthophosporic leading to long run time with broad peak, so the mobile phase containing 60 % orthophosporic acid was enough to give sharp peak with shorter run time. Furthermore, when orthophosphoric acid solution was substituted by other aqueous phases such as 0.1 % formic acid, ammonium acetate or potassium dihydrogen phosphate buffer, it led to inadequate resolution between DEM and its degradation product. Moreover, the mobile phases containing concentrations of 2, 5 and 10 mM of orthophosphoric acid were tried and the results indicated that, increasing the ionic strength led to the increase of the resolution time of DEM relative to its degrading and improved peak shape, so that 10 mM orthophosphoric acid was being chosen as an optimum concentration. Also, the mobile phase was pumped at a flow rate of 1.5 ml min^{-1} which was suitable for good separation within a reasonable time. Detection was tried at 254 nm, 225 nm and 210 nm, finally 225 nm was chosen as optimum measurements.

System suitability tests

The system suitability tests were performed to ensure that the proposed LC method was suitable for intended analysis. The parameters of these tests are column efficiency (number of theoretical plates), tailing of chromatographic peak, repeatability as % R.S.D of peak area for six injections and reproducibility as % R.S.D of retention time of a solution contain 60 µg ml^{-1} (100 % concentration) and 6 µg ml^{-1} of its degradant. These results are listed in table (1).

Table 1: System suitability tests for the proposed LC method.

Item	Data for DEM
N	2277
T	0.44
% R.S.D of six injections peak area	0.33
% R.S.D of six injection of retention time (min)	0.78
R	5.45

N: no of theoretical plates per column, T: tailing factor, % R.S.D: % relative standard deviation, R: resolution factor.

According to the previous results, the proposed method showed best resolution and peak symmetry with minimal elution time between DEM and its degradation product (DEG) using Eclipse XDB C_8 (250×4.6 mm, 5µm) column with mobile phase composed of 0.01 M orthophosphoric acid pH 2.6: acetonitrile (60:40) at ambient temperature. Also, the flow rate 1.5 ml min^{-1} was chosen as it reduced the run time without affecting resolution. Moreover, the detection based on peak area was conducted at 225 nm, where linear correlation was obtained between the peak area and the concentration. On the other hand, the system suitability parameters were measured in order to verify the system performance as shown in table (1) and the retention times for DEM and its degradation product (DEG) were shown in fig.(3).

Method validation

Linearity

In this study, eight concentrations were chosen for the cited drug. Each concentration was analyzed three times and linearity was studied for DEM. Linear relationship between area under the peak (AUP) and the concentration (C) was obtained. The regression equation for DEM was computed as shown in table (2). The linearity of the calibration curve was validated by the high value of correlation coefficient.

Accuracy

Accuracy of the results was calculated by % recovery of 5 different concentrations (injected in triplicates) of DEM in bulk powder and also by standard addition technique applied to pradaxa® 110 mg and 75 mg. The results obtained are displayed in table (2).

Table 2: Results obtained by the proposed LC method for the determination of DEM in the presence of degradation product (DEG).

Item	DEM
Retention time	3.238±0.2
Wavelength of detection	225nm
Linearity range	5-100µg mL^{-1}
Regression equation	y=47.377x-57.156
Sa	6.666441
Sb	0.118462
Regression coefficient (r)	0.9975
LOQ (limit of quantification)	0.23 µg mL^{-1}
LOD(limit of detection)	0.07 µg mL^{-1}
Drug in bulk (%)	99.65± 0.70
Standard added % (for 75 mg dosage form)	99.40± 1.118
Standard added % (for110 mg dosage form)	99.70± 1.13
Intra-day (%RSD)	0.13- 0.84
Inter-day (%RSD)	0.33-1.25
Drug in 75mg dosage form (%)	99.34± 1.36
Drug in 110mg dosage form (%)	100.2± 1.00

S_a: standard deviation of intercept, S_b: standard deviation of slope.

Precision

The repeatability (intra-day) was assessed by three determinations for each concentration (60, 75, 90 µg ml^{-1}) representing 80, 100, 120 % respectively of target concentrations. The values of precision (% R.S.D) of repeatability for DEM were found to be less than 1 % in the three concentrations (Table 3). Inter-day precision was evaluated through replicate analysis of 60, 75 and 90 µg ml^{-1} of DEM for three successive days. The values of precision (% R.S.D) for DEM were found to be less than 2 % (Table 4).

Specificity

Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences including degradation products, related substance and matrix components (Roy *et al*., 2014). In the present work, DEM was determined and separated from one of its related impurities namely dabigatran etexilate o-desethyl. Besides, the chromatogram of the sample was checked for the appearance of any extra peaks, no chromatographic interference from any degradation products or excipients was found at the retention time of examined drug (Fig. 3).

In addition, the chromatogram of drug in the sample solution was found identical to the chromatogram received by the standard solution. These results demonstrate that, the absence of interference from other materials in the pharmaceutical formulation and therefore confirm the specificity of proposed method.

Table 3: Results for determination of repeatability for DEM bulk powder by proposed LC method.

Conc. / Item	60 µg mL^{-1}	75 µg mL^{-1}	90 µg mL^{-1}
Mean	2938.87	3603.18	4254.62
Standard deviation	0.80	0.84	0.13
% R.S.D	0.81	0.84	0.13

% R.S.D: % relative standard deviation.

Table 4: Results for determination of inter-day assay for DEM in bulk powder by proposed LC method.

Item \ Conc.	60 µg mL^{-1}	75 µg mL^{-1}	90 µg mL^{-1}
Mean	2926.99	3626.74	4266.91
Standard deviation	0.933	1.09	0.33
% R.S.D	0.924	1.09	0.33

Limit of detection and limit of quantification

Limit of detection (ICH Q2B, 1996) which represents the concentration of the analyte at S/N ratio of 3 and limit of quantification (ICH Q2B, 1996) which represent the concentration of analyte at S/N ratio of 10 were determined experimentally for the proposed method and the results are shown in table (2).

Robustness

Robustness was performed by deliberately changing the chromatographic conditions. Only one factor was changed at a time while the other kept constant. The factor measured after each change was the resolution factor between the two peaks. Changing the flow rate of mobile phase by ±0.1, acetonitrile ratio by ±2 %, slight change in molarity of orthophosphoric acid and the column temperature by ±2ºC didn't have significant effect on chromatographic resolution by the proposed LC method for DEM and its degradation product, indicating good robustness of the proposed method as shown in tables 5.

Table 5: The Influence of different chromatographic parameters on the resolution of DEM from DEG.

Flow of mobile phase			
R	1.4 ml min^{-1}	1.5 ml min^{-1}	1.6 ml min^{-1}
	5.33	5.45	5.22
Acetonitrile strength			
R	38 %	40 %	42 %
	5.37	5.45	5.27
Molarity of orthophosporic acid			
R	9.62 mm	10 mm	10.38 mm
	5.37	5.42	5.29
Column Temperature			
R	23 oC	25oC	27 oC
	5.40	5.45	5.27

Statistical analysis

Statistical analysis of the results obtained by the proposed method and the company method was carried out by "SPSS statistical package version 11". The company method for DEM was based on mobile phase consisting of 0.2 % aqueous solution of ammonium acetate with dilute glacial acetic acid to pH 4.4 : acetonitrile by using gradient elution mode and Inersil ODSΠ (125mm×4mm, 5µm), detection was done at 340 nm and column temperature was 40ºC. The significant difference between groups was tested by one way ANOVA (F-test) and student t-test at p=0.05 as shown in table (6). The test ascertained that there was no significant difference among methods.

Table 6: Statistical comparison between the recovery results of proposed LC method and company method for DEM**.**

Drug	Group	n	Mean	S.D	t-value (1.782)*	f-value (4.77)*
DEM	Proposed method	5	99.65	0.70	=0.217	=1.978
	Company method	9	99.60	0.49		

**figures in parenthesis represent corresponding tabulated values for F and t tabulated at p=0.05*

Conclusion

The proposed LC method has the advantage of simplicity, precision, accuracy and convenience of separation and quantification of DEM alone or in the presence of its degradation product. Moreover, the proposed method is capable of simultaneous determination of DEM and it's degradant in laboratory prepared mixture and pharmaceutical formulation. Therefore, the proposed LC method can be used for quality control of the cited drug.

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