SPECTROFLUORIMETRIC DETERMINATION OF EOSIN CHEMILUMINESCENCE SYSTEM FOR THE ANALYSIS OF DABIGATRAN IN PHARMACEUTICAL FORMULATIONS

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ABSTRACT

Objectives: The objectives of this study were to develop a rapid, simple, and economical spectrofluorometric method for quantification of dabigatran from marketed formulation and its principle involved based on the protonated process of a binary mixture complex formation with eosin Y.

Methods: A simple and precise spectrofluorometric technique was applied for the method development. It depends on measuring the quenching effect of the drug on the native fluorescence of eosin at excitation under the optimum reaction conditions.

Results: The reaction linear calibration graph constructed between the fluorescence quenching valves flouresence intensity (ΔF) and the concentration ranges of 5–50 µg/ml. Spectrofluorometric analytical performance was validated by accuracy, precision, and specificity, and the results were satisfactory.

Conclusion: This method was to develop a fast, simple, and economically applied successfully for the assay, and qualification of dabigatran tablet contains drug, also with different coformulated pharmaceutical formulations.

Keywords: Spectrofluorometric method, Dabigatran mesylate, Eosin Y.

INTRODUCTION

Dabigatran is an aromatic amide obtained in thermal condensation of a carboxy group, chemically dabigatran (2-[{(4-carboxylic phenyl) amino) methyl 1)-1-methyl-1H benzimidazole-5-carboxylic acid with the secondary amino group of –N-pyridine-2yl-beta-alanine. The active moiety of prodrug dabigatran mesylate, it uses with coadministration of P-glycoprotein inhibitor risk of developing adverse drug reactions such as kidney dysfunction and high body mass index [1,2].

Need of the study

A literature search revealed only few quantitative analytical methods for the estimation of dabigatran. Further, few methods only described the quantification of dabigatran in biological fluids, as these methods include liquid chromatography–*mass spectrometry* (LC–MS) with require expensive instruments not available in the conventional bioanalytical laboratory. Dabigatran high-performance LC–MS/MS methods affected the sensitive for analysis of dabigatran in biological fluids [3,4]. However, their sophisticated instrumentation and high cost of analysis limit their use for analysis of drugs. At through, the spectrofluorometric is a highly specified sensitive and simple technique [5,6]. However, up to now, no method has been reported in the spectrofluorometric determination of dabigatran. Eosin Y is known to react in the primary functional groups of alcohols and amines, imidazole's, and thiols. Hence, eosin Y has been used as a fluorogenic reagent to the determination of many pharmaceutical compounds [7,8]. In this present study, we devloped chemiluminesence system quantification technique following by dabigatran binary complex formation has been based on its reaction; photon-induced reduction of eosin Y in acetate buffer (pH 4.0) solution. The free fluorescent producer which is measured by fluorimetrically at (329 nm to 519 nm). Hence, we develop a fast, simple, and economical method for quantification of dabigatran from the marketed formulation. The developed method was validated as per under the US FDA guidelines.

METHODS

Dabigatran mesylate was kindly supplied by Madras Pharma (Chennai); eosin Y and carboxyl methylcellulose (CMC) (Fine Chem Industries, Mumbai - 600 005), sodium acetate (Lab Chem Pvt., Mumbai), orthophosphoric acid (OPA) (Thermo Fisher Scientific Pvt., India), and eosin Y (0.5×10^{-4}) M aqueous solution were freshly prepared in distilled water. Acetate buffer solution was prepared by mixing an approximate volume of 2×10^{-3} M sodium acetate, and 0.1 M OPA – adjusting to pH to 4.0 using pH meter. Carboxymethyl cellulose was prepared as 0.50% w/v by dissolving the small amount in hot water (80°C) with continuous string (magnetic stirrer) 15 min and then chilling to 5°C for 80 min. All the materials and reagents were of analytical grade and all solutions were prepared with distilled water.

Pharmaceutical formulation

Pradaxa-110 mg and 150 mg capsules Anteka Healthcare Private Limited (New Delhi).

Preparation of dabigatran mesylate standard solution

A stock solution of dabigatran mesylate was prepared by weighing accurately 10 mg of drug transferred in clean dried 10 ml volumetric flask and adds an approximate volume of water and sonicates 15 min and completed to 10 ml with distilled water. The marking standard solution was prepared by further dilution of the stock solution with the same solvent to obtain concentration required range. The solution was stable at least 1 week when kept in the refrigerator.

Preparation of sample

Dabigatran mesylate marketed sample was prepared by weighing accurately 80 mg of drug transferred in clean dried 10 ml volumetric flask and adds an approximate volume of water and sonicates 30 min make up to the volume. The solution passed through a 0.45 µm syringe filter then the sample solution using further dilution with the same solvent to obtain concentration required range.

General recommended procedure

One milliliter of sample or standard dabigatran solution concentration (5–50 µg/ml) was transferred into a 10 ml calibrated volumetric flask, followed by 1.0 ml of eosin solution (5.0×10^{-4} M), 0.50% w/v of CMC solution, and 1.0 ml of 2.0×10^{-3} acetate buffer (pH – 4.0). The volumes

were completed up to the mark with distilled water. The emission of the solution was measured at 519 nm after excitation at 339 nm. The difference in the ΔF was plotted versus the drug concentrations.

Procedure for validation of the proposed

Construction of calibration

The dabigatran mesylate standard solution having a different concentration (5–50 µl/ml), the fluorescence intensity (ΔF) was measured for each concentration and the calibration graph was constructed between concentration and ΔF. Analytical parameter such as correlation coefficient, slope, and intercept was calculated and applying to the linear regression equation. A limit of detection (LOD) was calculated using LOD = $3\sigma/s$, which limit of quantitation (LOQ) = $10 \sigma/S$, where σ is the standard deviation of intercept and S is the slop or calibration curve.

Accuracy and precision

In this method, repeatability was investigated by applying the general recommendation procedure using and replicated (5 µl/ml). Respectively, intraday precision was performed by carrying the analysis at the 3 successive times within the same day. Moreover, intermediate (interday) precision was calculated using three replicated at the 3 different environmental successive days.

Determination of molar ratio of the reaction between dabigatran end eosin Y

Breifly, stoichiometry of the reaction Estimated by job's method following by continues variation under the working condition. Master solution of equimolar solution eosin Y (1.5×10^{-4}) and dabigatran was prepared. Series of 1:0 ml portions of the master solution of eosin Y and dabigatran were up comprising different complementary proportions (0.1:0.9, 0.2:0.8….0.9:0.1, 10:0) in 10 ml volumetric flasks. Add 1.0 ml of carboxylmethyl cellulose surfactant (0.50% w/v) and 0.5 ml of acetate buffer (pH 4.0) was added. The volume was completed to 10 ml with distilled water. In the spectrofluorometric method, fluorescence intensity measured at 519 nm against reagent blank treated similarly. The emission of the solution was measured 519 nm after excitation of at 339 nm.
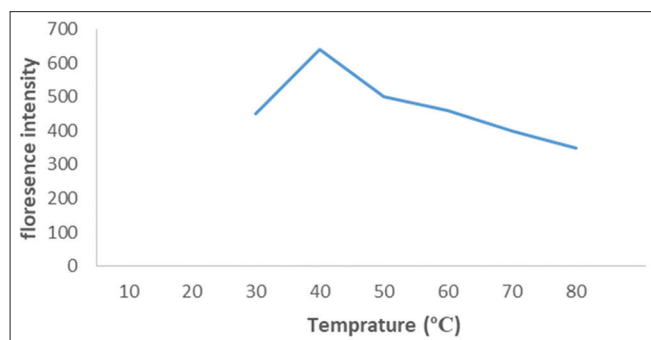

Fig. 1: Effect of temperature in the development reaction of dabigatran and eosin Y
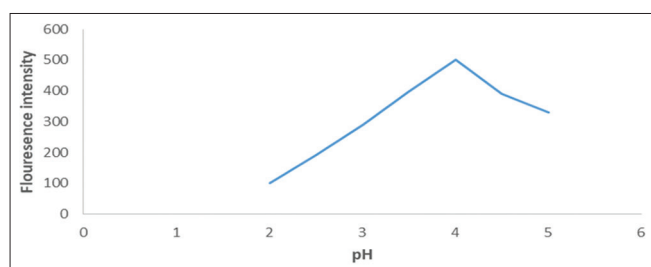

Fig. 2: Effect of pH on the development reaction product of dabigatran and eosin Y

RESULTS AND DISCUSSION

Binary mixture complex formed between basic (dabigatran) and eosin Y. The formed complex is low water solubility ion-pair association complex. It was usually extracted with different organic solvents such as methyl tert-butyl ether and dichloromethane. These extraction steps are no formation of the ionic complex. Since alternatively choose surfactant. The surfactant greatly enhanced the water solubility of the complex formation. Hence, this method is aqueous solution no need for any extraction step and direct measurements in this present work, CMC was used a surfactant to improve the wetting solubility of the formation of ion-pair complex. The present study is based on the nitration of dabigatran and eosin Y at pH (4.0); the formed complex is mainly due to the electrophilic interaction between the 3° amino groups in dabigatran and anionic functional group of eosin under acidic pH (4:0). The formed ion-pair complex is not fluorescent, but the formed complex is accompanied by eosin Y which is decreased in the native fluorescence intensity at 519 nm at emission 339 nm [9,10].

Optimization of reaction conditions

Various factors affect the formation and stability of the binary complex of eosin Y and dabigatran. The proposed method studied and optimized for the development of the assay procedure. The studied factors induced temperature, concentration of eosin Y, pH, and the dilution solvents.

It was focused that by increasing the reagent concentration. The ΔF was gradually increased. The maximum obtained ΔF results in eosin Y (1.0×10^{-4} M).

The temperature influence of fluorescence quenching of the binary complete was studied over the temperature range (10–90°C), as shown in Fig. 1.

The complex formation was greatly attended by temperature maximum ΔF values, was obtained that the stable temperature of the reaction mixture was 40°. The influence of pH on the ΔF quenching of the binary ion-pair complex was studied pH range (2–7) using the acetate buffer, as shown in Fig. 2.

The complex formation was greatly increased pH of the maximum (ΔF) which was obtained that the pH of the reaction is 4.0 at 40°C. The reaction between the drug and eosin Y was completed immediately after mixing of the solution measure (ΔF) at emission 519 nm and excitation 339 nm remain constant. The formed complexing was found to stable for at least 48 h after dilution, as shown in Fig. 3.

The formed chromogen was diluted with different solvents such as water, ethanol, dichloromethane, and methanol. The water was found to be the most appropriate solvent for dilution. Other study solvents giving lower results (Table 1).

Stoichiometry and mechanism of the reaction

The nature of the binary complex between eosin Y and dabigatran mesylate has been determined by applying the job's method of continuous variation. The concentration reached to the maximum value at a mole fraction of about 1.0, which indicated the formation of a 1:1

Table 1: Maximum excitation and emission wavelength of the binary product (dabigatran/eosin Y) with extract in different organic solvents at concentration range (1000 ng/ml)

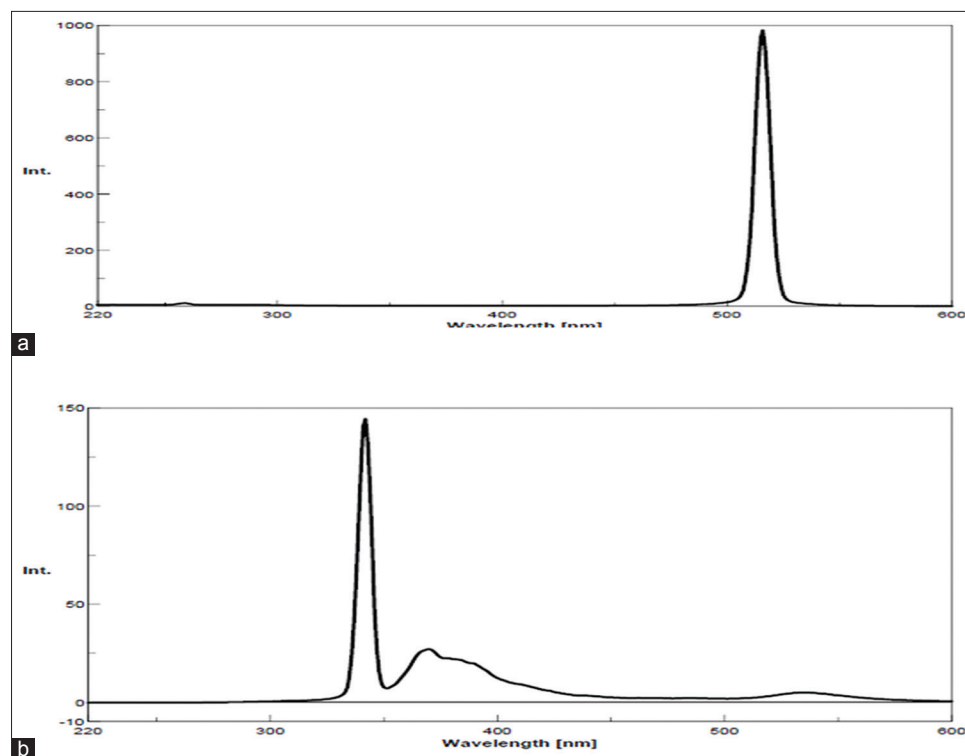| Extraction solvent	Λex/λem (nm)	Fluorescence intensity (ΔF)
Dichloromethane	378/491	490
Carbon tetrachloride	360/497	497
Methyl tart-butyl ether	385/486	486
Methanol	354/489	490
Water	339/519	519

Fig. 3: Binary mixture of dabigatran emission spectrum (a) and excitation spectrum (b)

drug-dye complex depending on the pH (4.0) of the solution [11,12] Fig. 4.

Eosin (O) denotes the anionic part. The pKa1, pKa2, and pKa3, in the presence of molar concentration, were 2.50, 3.20, and 4.50, respectively. In pH 4.0 weak acidic medium, eosin Y exists mainly in the monovalent anionic form. There are two possibilities for the ionization of eosin, by dissociation of the hydroxyl or carboxylic groups. In eosin analog without bromo substitutions, the carboxylic group is first ionized then followed by a hydroxyl group. The eosin presence of two strong electron-withdrawing bromo groups close to the hydroxyl group reduces the charge density at the oxygen atom of the hydroxyl. Hence, the hydroxyl group tends to dissociate more easily than the carboxylic group. Therefore, eosin monovalent anion is formed by the ionization of the hydroxyl group. Dabigatran has a tertiary amino group that is easily protonated in acidic medium to form a positively charged cation. The ion association complex is formed by the interaction of the protonated tertiary amino group of dabigatran the ionized hydroxyl group of the eosin monoanionic through electrostatic attraction and the hydrophobic forces. The ionic reaction complex formation was confirmed by IR spectra [13,14] (Fig. 5). IR spectrum did not provide more information since several absorption bands for the stretching of O-H and N-H bonds were observed in the same regions. However, the extinction of characteristic O-H signals at 1284 cm^{-1}, in the IR spectrum of the reaction product provided that one eosin Y moiety was attached to the drug molecule at hydroxyl group (Fig. 5).

METHOD VALIDATION

Quantification, linearity, accuracy, and precision
The optimum reaction conditions and calibration curves were constructed and the analytical parameters were calculated. The LOQ and LOD were calculated according to the ICH guidelines. The present study optimized chromatographic conditions shown in (Table 2).

Linearity
One milliliter of sample or standard dabigatran solution concentration (5–50 μg/ml) was transferred into a 10 ml calibrated volumetric flask,

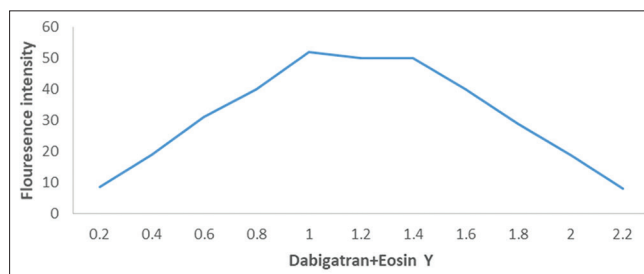**Fig. 4: Molar ratios obtained for the reaction of eosin Y with dabigatran using molar concentration of 1.0×10^{-4} M**

Table 2: Analytical parameters for the analysis of dabigatran the proposed method

Parameters	Obtained values
Concentration range (μg/ml)	5–50
Intercept	18.43
Slope	77.60
Correlation coefficient (r^2)	0.9778
λex/λem (nm)	318/519
SD of slope	1.07
SD of intercept	1.54
LOD (μg/ml)	0.355
LOQ (μg/ml)	0.813

SD: Standard deviation, LOD: Limit of detection, LOQ: Limit of quantification

followed by 1.0 ml of eosin solution (5.0×10^{-4} M), 0.50% w/v of CMC solution, and 1.0 ml of 2.0×10^{-3} acetate buffer (pH – 4.0). The volumes were completed up to the mark with distilled water. The emission of the solution was measured at 519 nm after excitation at 339 nm. The difference in the ΔF was plotted versus the drug concentrations, as shown in Fig. 6.

Precision
Precision studies were estimated at two levels, repeatability and intermediate precision through five replicate analyses of the drug
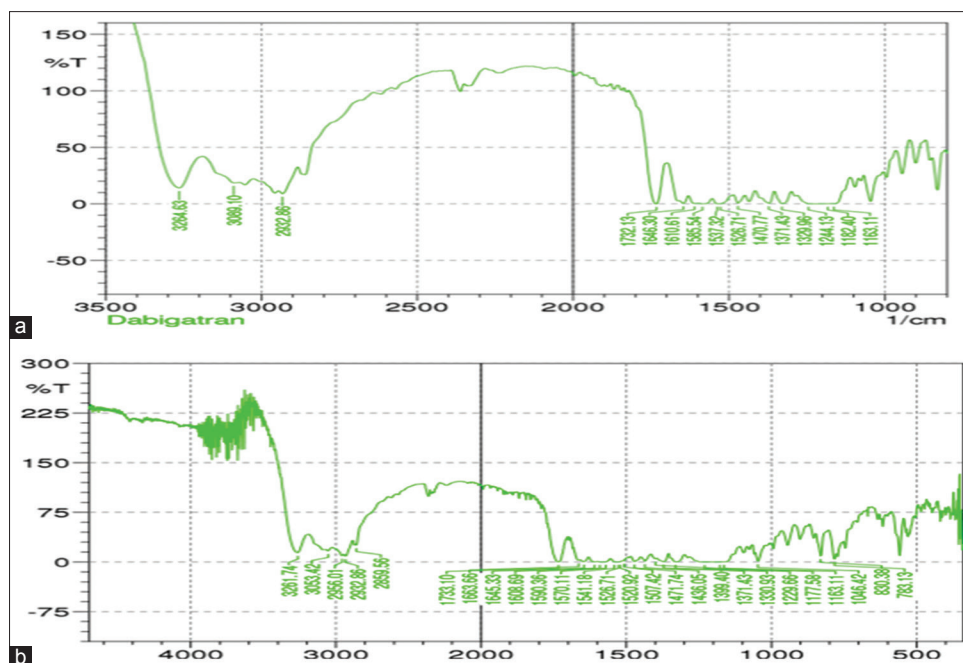
Fig. 5: IR spectrums of the dabigatran and binary complex compound. (a) Standard IR spectrum of dabigatran. (b) Standard IR spectrum of the binary complex
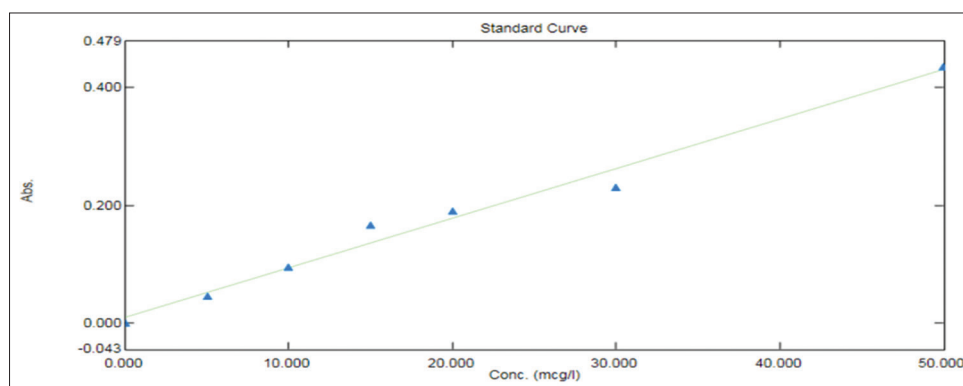

Fig. 6: The linearity spectrum of dabigatran and eosin Y complex

Table 3: Intra- and interday assay of precision of the proposed method for the determination of dabigatran

Precision	Recovery (%) ± SD	RSD (%)
Intraday precision Mean±SD	98.58±0.21	1.12
Interlay precision		
Day 1	99.8±0.81	0.699
Day 2	100.83±0.27	0.831
Day 3	100.56±0.44	1.01
Mean±SD	99.94±0.25	

The value of the average of three determinations in proposed method (n=3).
SD: Standard deviation, RSD: Relative standard deviation

(0.5–10 µg/ml). The repeatability (intraday precision) was performed by the analysis 3 successive times within the same day; intermediate (interday) precision was performed at 3 successive days. The low values of standard deviations indicated high precision of the proposed methods; the obtained dates are shown in Table 3.

Specificity

The specificity studies of the method were investigated by observing dabigatran marketed condition. The sample was prepared by weighing

Table 4: Specificity analysis of commercial tablets the proposed method

Product	Mean±SD	Recovery (%)±SD	T	F
Pradaxa 110 mg	110.59±0.41	100.59±0.49	0.26	0.49
Pradaxa 150 mg	150.74±0.26	100.47±0.19	0.68	1.85

The value of the average of three determinations in proposed method (n=3).
SD: Standard deviation

accurately 80 mg of drug transferred in clean dried 10 ml volumetric flask and added approximate volume of water and sonicated 30 min make up to the volume, then after passed through 0.45 µm syringe filter. After suitable dilution, the obtained solution was analyzed by proposed methods. Obtained dates are shown in Table 4.

CONCLUSION

The progression of chemiluminesence spectrofluorometric quantification technique of dabigatran. These methods are simple, sensitive, and environmentally safe since water is the reaction solvent which is the most important green solvent, organic solvents are omitted in the present work and eosin Y is a less toxic regent. This method is not using expensive solvents and instruments, there is simplicity, the

sample is extracted with aqueous no need any organic solvents, and the formed eosin Y as an ion-pairing complex was directly measured in the aqueous solution. The devloped method are sensitivity , simplicity and applied for suitable routine analysis of dabigatran marketed formulations.

ACKNOWLEDGMENTS

AUTHORS' CONTRIBUTIONS

D.Y and M.W conceived the study and design the experiments. D.Y. performed the experiments and analyzed the data; S.V. wrote the manuscript.

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