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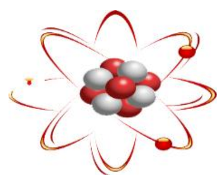

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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DABIGATRAN IN HUMAN PLASMA BY REVERSE PHASE –HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION IN BIOEQUIVALENCE STUDIES

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ABSTRACT

A new sensitive, accurate, rapid and reproducible HPLC-UV method for determination of dabigatran in spiked human blood plasma was developed and validated. The Analyte Dabigatran and internal standard (promethazine) were extracted with Methyl tert-butyl ether. The chromatographic analysis was performed on a sun fire C18 column (4.6x150mm) 3.5µm using acetonitrile phosphate buffer 40:60 v/v (p H 3.0 OPA) as a mobile phase at a flow rate 1ml/min andthe detection was carried out at 222nm. The linearity range was observed in the concentration range 50-1500ng/ml and the heteroscedasticity was minimized by using weight least squares regression with weight factor $1/_x$. The inter and intraday % RSD was found to be within limit. Results of recovery studies prove the extraction efficiency. The stability data indicated that dabigatran was stable in plasma after three freeze thaw cycles and room temperature upon storage at -20ºc for 1 month. The proposed method was successfully applied in Bio equivalence studies.

Keywords: Dabigatran, Promethazine, Human spiked plasma, RP-HPLC determination.

INTRODUCTION

According to WHO reports, leading global cause of death is due to related to cardiovascular disease (3%). the total global incidence of Atrial fibrillation (AR) associated with CVD is (1-2%), warfarin is used successfully treat of CVD, warfarin induced harmful adverse drug reaction like, narrow therapeutic window, drug and food interactions, substrate for various isoforms, of cytochrome p450 (CYP and variable protein bindings) [1, 2]. Then after developed to the novel oral anticoagulant molecule dabigatran. Thenovel anticoagulant most promising drug class for treating CVD, the mechanismof action direct thrombin (factor 2 and 2a) inhibitor, dabigatran was registered in 2010 by the US-FDA[2,3].

Dabigatran is an aromatic amide obtained in thermal condensation of carboxy group , chemically dabigatran (2-[{(4-carboxylic phenyl)amino)methyl 1)-1-methyl-1H benzimadazole-5-carboxylic acid. with the secondary amino group of –N-pyridine-2yl-beta-alanine [3, 4]. The active moiety of prodrug dabigatran Mesylate. Dabigatran its use poses the risk of developing ADR, due to the age and physiological factors [5, 6], with co-administration of p-glycoprotein (p-gp) inhibitor. The therapeutic drug monitoring was reported case of bleeding during the perioperative period and with co-administration p-gp inhibitor. Kidney dysfunction and high body mass index etc...[7]. A literature search revealed an only few quantitative analytical method for estimation of dabigatran. Further few methods only described the quantification of dabigatran in biological fluids, like these methods include LC-MS with require expensive instruments not available in conventional bioanalytical laboratory [8]. Hence decided to develop a fast, simple and economical method which was based on simple sample extraction process. And HPLC with UV detection for quantification of dabigatran from spiked human plasma [9, 10]. The developed method was validated as per US FDA guidelines.

MATERIALS AND METHODS

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Chemical and reagents

The reference standard of Dabigatran mesylate was received as a gift sample from Madras pharma Limited, Chennai. HPLC grade Acetonitrile and Trimethylamine were purchased from Sigma Aldrich, India. O-phosphoric acid was purchased from Thermo Fischer pvt ltd.Ultra-pure water was obtained from Mille-Q water purification system from Millipore (Milford) USA.

Instruments

Chromatographic separation was achieved using HPLC waters system which consist of waters pump control module-II,waters 515 solvent delivery system (pump),Rheodye injector (20µ loop),waters 2489 UV-Visible detector,Empower2 software from waters corporation as data processor.Analytical column sun fire C18(4.6x150mm)3.5µm.The mobile phase was passed through a 0.45 µm membrane filter and degassed by using Sonicaultra-sonic cleaner by spincotech Pvt ltd .Apart from this Sartorius single pan digital balance (BSA 224S-CW) was used for weighing the samples. Arson 7596 vortex mixer used for mixing purpose of samples.Ultra sonicator cleaner wasused for proper dissolving as well as mixing of samples.Shimadzu 1600 pc UV double beam spectroscopy was used for analysing λmax of the selected components.Lab India controlled pH analyser was used to check the pH of the mixtures.

MOBILE PHASE PREPARATION

Buffer preparation (2Mm)

Accurately weighted and transferred about 0.273g of potassium dihydrogen ortho phosphate into 1000ml volumetric flask. To this add 1000mlof mille Q water and 1.0ml of triethyl amine. The pH was adjusted to3.0 using ortho phosphoric acid. Then the buffer was filtered by passing through 0.4µm membrane filter, degassed and stored.

Preparation of mobile phase

The mobile phase was prepared by mixing buffer and Acetonitrile in (400:600 v/v) ratios.

Preparation of calibration curve (cc) standards and quality control (QC) samples

Preparation of standard stock solution

A standard stock solution of dabigatran (1000µg/ml) was prepared by dissolving 10mg of dabigatran using small quantity of acetonitrile in10ml volumetric flask, and make up to volume with water and sonicates to10mins.

Serial dilutions of standard stock solution of dabigatran (1000µg/ml) was carried out to get working standard solutionof concentrations 5, 10, 20, 50, 80µg/ml. Aliquots of 1ml of human blank plasma were spiked with 20µl of the working standard solutions to get the CC standards containing 50, 100, 200, 300, 600, 1200ng/ml of dabigatran. Thesimilarly QC samples were prepared containing 300ng/ml LQC and 900ng/ml MQC, 1800ng/ml HQC.

(IS) Standard stock solution

The stock solution (1mg/ml) of promethazine was prepared in water and then appropriately diluted with water to get working standard solution 250µg/ml.

Sample preparation

1 ml of human plasma spiked sample, and 5ml methyl Tert-butyl ether was added to glass tube containing working standard solutions and 1 ml (internal standard) the contents of the tubes were, mixed on a vortex mixer for 1min. Then the tubes were centrifuged at 3500 rpm for 15min. From this 3ml of the organic layer was pipette out into separate tube and evaporated to dryness under s stream of nitrogen. The residue was reconstituted with mobile phase and subjected to chromatographic analysis using the optimized chromatographic conditions.

Chromatographic conditions:

The optimized parameters which were used as a final method for estimation of dabigatran mesylate represented in the Table.1.Fig 1: Shows UV spectrum of dabigatran mesylate. (A wave length 222nm).

Calibration studies

The calibration curve was constructed by analysing 5 samples of intact human blood plasma with added dabigatran and internal standard solution to get concentrations of 50, 100, 300, 600, 1200ng/ml. The data of concentrations and the corresponding area ratio of dabigatran to internal standard were subjected to weighted least square linear regression. Theequation generated were used to calculate interpolated concentrations, % relative error (%RE) was calculated for the each CC standards. Fig 2: shows the obtained calibration curve and correlation coefficient.

$$\% RE = \frac{\text{interpolated concentration} - \text{nominal concentration}}{\text{Nominal concentration}} * 100$$

Method validation

The validation of developed method was conducted according to the US FDA guidance for industry (2008).

Bioanalytical method selectivity was studied by analysing specificity of five samples intact with human plasma from various sources, the lower limit of quantification (LLOQ) of 250ng/ml by comparing blank response of plasma peak areas.Afforded by the LLOQ samples. Accuracy and precision were studied by analysing five replicates of the lower quality control (LQC), middle quality control (MQC), and higher quality control (HQC) over 1 week. Theconcentration of

dabigatran QC samples were determined by referring to the area ratio of the drug to internal standard, obtained to the QC samples. Theregression equation generated on the same day. The accuracy was estimated as the mean %RE while the precision was measured in terms of % RSD.

Percentage recovery of the extraction procedure was calculated by comparing the peak areas of the processed QC samples. Stability studies of the dabigatran in human plasma was determined under various conditions. Namely three freeze thaw stability cycles, stability at room temperature, for 6 h, and short term stability at -20°c for 1 month. The determination of freeze thaw stability, five replicates of the LQC andHQC samples were frozen -20° c for 24h. Andallowed to thaw unassisted at room temperature. The short term room temperature stability, five replicates of LQC,and HQC samples place at room temperature for 6h. A short term stability at -20°c was determined by using five replicates of LQC,and HQC samples kept for at -20°c for 1month. The amount of the drug stability samples was find out the % of nominal and %RSD were calculated.

Data analysis

Data are reported as mean ± standard deviation (SD) for the replicates. The(RSD) values and accuracy were calculated using Microsoft excel (2003).

RESULTS AND DISCUSSION

Chromatographic determination was selected after several trials after on different column usingdifference mobile phases comprised of acetonitrile and phosphate buffer at varying pH and in different proportions.Separation was done by using sun fireC18 (4.6x150mm) 3.5µm. 2.7µm and mobile phase of acetonitrile phosphate buffer (40; 60 v/v) at a flow rate 1ml/min was found to be most suitable to attain adequate resolution and satisfactory peak shapes for dabigatran and internal standard. The detection of the drug was monitored at wavelength at 222nm was chosen for maximum sensitivity. The liquid- liquid extraction was performed used different solvents like diethyl ether, c dichloro methane, ethyl acetate, methyl tert-butyl ether.It was observed that both drug and internal standard were appreciably extracted with methyl tert-butylether. The extraction recovery for dabigatran 75-80% and promethazine 76-80%. Methyl tert-butyl ether is less polar and lighter than other solvents; it is less likely to extract interferents and is easily transferable. Somethyl tert-butyl ether was selected as the extracting solvent. During the calibration experiments of the standard deviation of area ratios of CC standards increased with concentration indicating a need for weight regression. Thus, weighted linear regression with weighing factor 1/x was selected as the calibration model, thus result in the equation y= 16851 x+603984 .The area ratios of calibration experiments depicted in Table 2. The validation studies during before it was found the peaks areas for the LLOQ samples more than five replicates of the blank responses obtained using the six different plasma sources it gives in Table 3. This method proved was selective at the LLOQ 250ng/ml. the blank plasma chromatogram show in fig 3. Shows the lack of significant interference at the retention times of dabigatran and internal standard. The representative chromatogram of MQC sample is shown in fig 4. The% RE and RSD% should be less then ±15 % requires for the US-FDA guidance. The results of assay precision, accuracy and extraction efficiency are shown in Table 4.the degrees of extraction of dabigatran and promethazine, during sample preparation calculated the ratio of peak area of the studied compounds. The chromatogram of spiked plasma with added working standard solution before precipitation and added working solution in spiked plasma in after protein precipitation the degree of extraction was determined for dabigatran at are tabulated concentration 250ng/ml and 3000ng/ml and promethazine 4.5ng/ml the results representedin Table 5 and 6.The results stability evaluation of dabigatran are presented in Table 7. The analysis of LQC and HQC samples subsequent to various stability cycles, three freeze-thaw cycles, stability at -20c for 1 month and stability at room temperature for 6h indicated for dabigatran was stable in human plasma under these conditions.

Table 1. Optimized parameters of chromatographic separation conditions

Optimized chromatographic conditions Optimized chromatographic conditions	
Column	Sunfire C18(4.6x150mm)3.5µm
Flow rate	1ml/min
Wavelength	222nm
Temperature	40c
Injection volume	20µl
Mobile phase	Acetonitrile : buffer (6:4)
PH of mobile phase	3.0 with OPA
Mode operation	Gradient
Run time	8 min

Table 2. Area functions ratios from calibration experiments

S.No	Amount of drug ng/ml (n=6)	Area Ratio (mean ±SD, n=6)
1	50	0.24 ± 0.01
2	100	0.53± 0.01
3	200	1.40± 0.05
4	600	3.64±0.13
5	1000	4.95± 0.11
6	1200	6.87 ±0.13

Table 3. Blank response and peak area ratio LLOQ of dabigatran

S.No	**Blank Response (mAUsec)**	**Peak Area at LLOQ (mAUsec)**
1	7.75	13976
2	7.75	13962
3	7.62	13929
4	7.64	13889
5	7.57	13839
6	7.67	13919

Table 4. Results of accuracy and precision studies of dabigatran

Level	Concentration added (ng/ml)	Intra-day (n=5)			Inter-day(n=5)			Recovery (n=5)
		mean concentration (ng/ml)	RE %	RSD%	mean concentration (ng/ml)	RE %	RSD%	
LOQ	250	254.67	2.31	6.24	255.48	2.23	5.46	80.45
MOQ	3000	2984.36	-3.2	5.91	2952.56	-4.6	7.6	82.21
HOQ	8000	8141.76	4.71	6.51	8080.48	2.36	5.86	76.35
IS	_							80.75

Table 5. Dabigatran degree of extraction

Parameter	Dabigatran concentration			
	100ng/ml		**300ng/ml**	
	A	B	A	B
Average peak area (n=5)	41232	44362	347251	353716
Degree of extraction (A/B)	0.92		0.98	

Table 6. Internal standard degree of extraction

Parameter	Internal standard concentration	
	4.95ng/ml	
	A	B
Average peak area (n=5)	75773	74896
Degree of extraction (A/B)	1.01	

Table 7. Results of stability studies of dabigatran

QC level	**Stability at Room temperature**		**Stability at -20°C**		**Freeze thaw-stability**	
	% nominal	**(%) RSD**	**% nominal**	**(%) RSD**	**%nominal**	**(%) RSD**
LOQ	102.87	4.13	92.43	8.9	86.23	4.7
HOQ	105.68	6.9	102.89	11.1	88.34	7.6

Fig 1. UV spectrum detection wavelength of dabigatran mesylate. (A wave length 222nm)

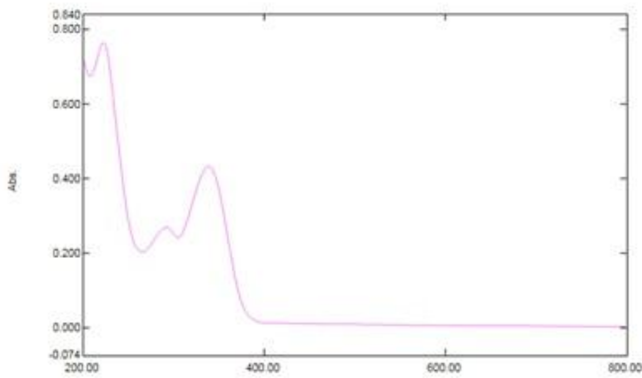


Fig 2. Calibration curve for peak area ratio of dabigatran and internal standard as a function of concentration ratio dabigatran and internal standard.

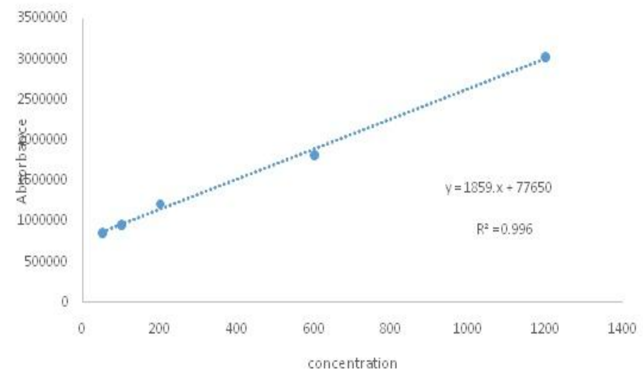


Fig 3. Representative HPLC chromatogram blank plasma extract (a wavelength = 222nm)

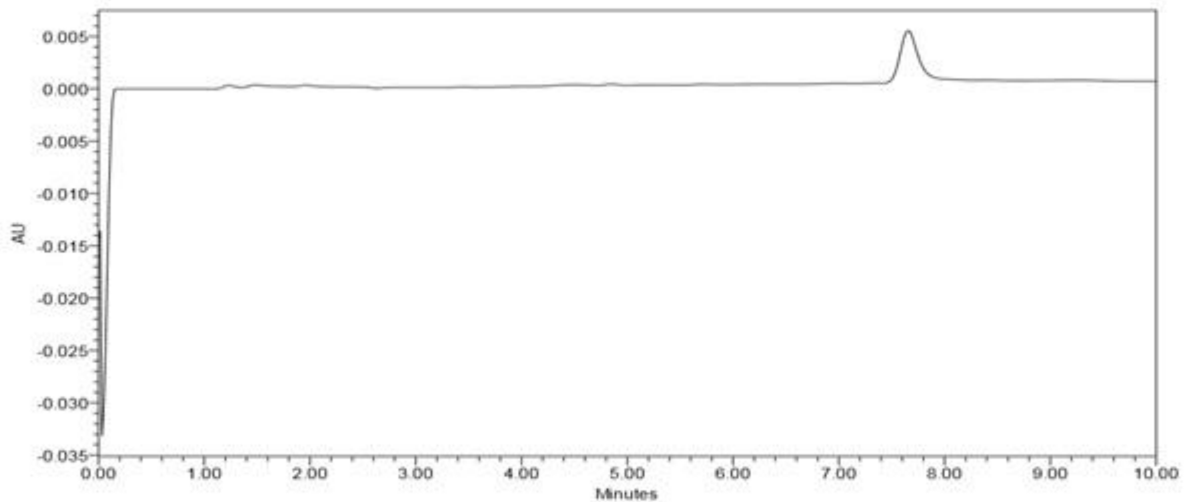
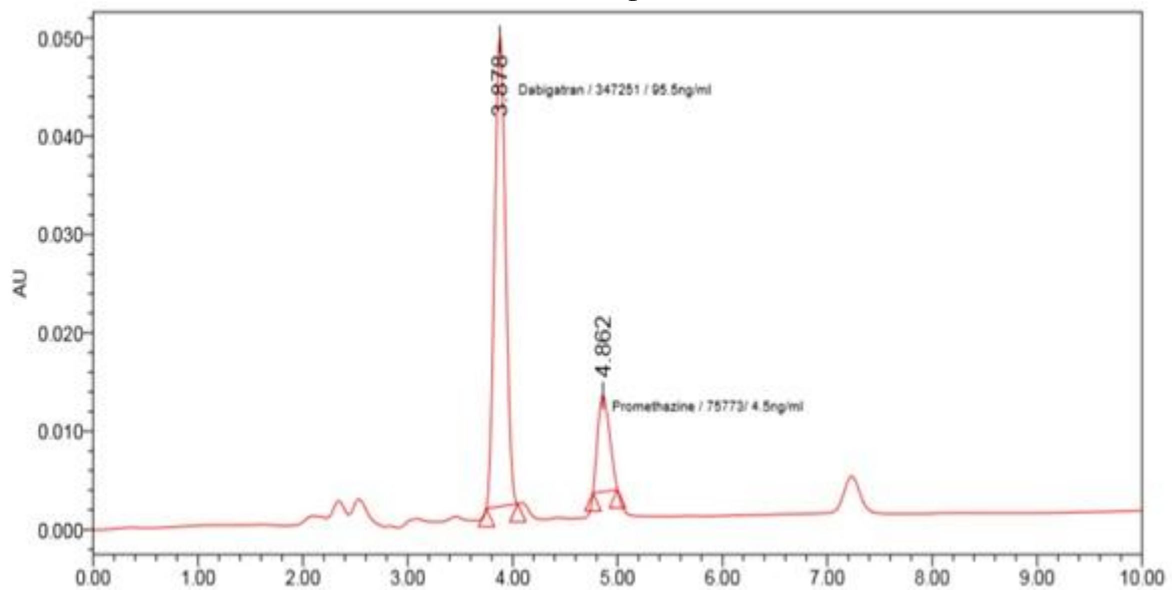


Fig 4. Representative HPLC chromatogram MOQ sample of dabigatran and internal standard, showing dabigatran (RT 3.878min) and internal standard (RT 4.862min) (a wavelength = 222nm)



CONCLUSION

A simple, rapid, sensitive, precise and economical HPLC-UV method with PDA detector was developed and validated for determining dabigatran in human spiked plasmaas per US FDA guidelines.The method reported here does not require expensive chemical and solvents, and also use a simple and effective extraction technique with good and reproducible recovery.This proposed HPLC method can be used for bioanalysis of dabigatran from plasma support bioavailability bioequivalence studies.

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CONFLICT OF INTREST

No interest.

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