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Original Article

Development and validation of bio-analytical method for the quantitative estimation of rivaroxaban by using UV spectrophotometry

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ABSTRACT

The present study deals with the development and validation of bioanalytical method for the quantitative estimation of rivaroxaban by using UV spectrophotometric technique. Maximum absorption of 250 nm, was selected for the analysis and the drug obeyed Beer-Lambert's law in the concentration range of 1000-7000ng/ml. Coefficient of correlation for linearity was found to be 0.995. The developed method has been validated for accuracy and precision as per USFDA guidelines. LLOQ, LQC, MQC and HQC values were found to be within the acceptable limits. The developed method was successfully applied for determination of rivaroxaban in spiked rat plasma with good recovery.

Key words: Rivaroxaban, USFDA guidelines

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INTRODUCTION

Rivaroxaban is an orally active direct inhibitor of activated factor Xa which became available for prophylaxis and treatment of DVT. Its anticoagulant action develops rapidly within 3-4 hours of ingestion. It is largely metabolized, but also excreted unchanged in urine, plasma t1/2 is 7-11 hours.[1]

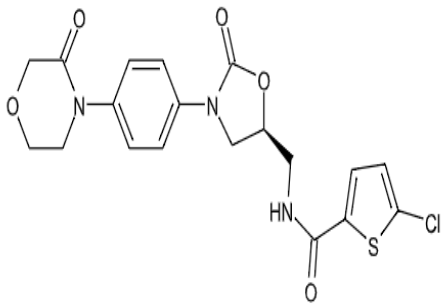


Figure: Structure of rivaroxaban

UV-Visible spectrophotometry is used for qualitative and quantitative analysis which involves measuring the amount of ultraviolet or visible radiation absorbed by a substance in solution.

A bioanalytical method is a set of procedures involved in the collection, processing, storage, and analysis of a biological matrix for a chemical compound. Bioanalytical method validation (BMV) is the process used to establish that a quantitative analytical method is suitable for biomedical applications. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use.[2,3]

MATERIALS AND METHOD

Materials: Acetonitrile, Rivaroxaban (pure drug) and Rivaroxaban tablets (Xarelto 20mg).

Method development:

Selection of solvent: Sample solubility was checked in different solvents like methanol, 0.1N NaOH, 0.1N HCl, phosphate buffer pH-6, 4 and acetonitrile. Sample is soluble in acetonitrile and insoluble in remaining solvents.

Preparation of stock solution I (1000µg/ml): 10mg drug weighed and transferred into 10ml volumetric flask. To this added few ml of solvent acetonitrile and sonicated for few minutes and made up with solvent up to mark.

Preparation of stock solution II (100µg/ml): 1ml of stock-I solution was transferred into another volumetric flask and made up the remaining portion with solvent up to mark.

Determination of absorption maxima (λ_{max}): 90µl of plasma was taken into volumetric flask and transferred 1ml from stock-II solution and made up the remaining portion with solvent up to the mark which gives 10µg/ml. The solution was scanned in the range of 200-400nm. The λ_{max} was found to be 250nm. The spectrum is shown in fig no. 01

Experimental animals: Male albino wistar rats (180-200g) were obtained from National Institute of Nutrition, Hyderabad

Plasma sample preparation: The blood samples were collected from retro- orbital puncture into disodium EDTA vials (20mg disodium EDTA in 1ml water, 1ml of blood requires 50µl of disodium EDTA) plasma was separated from blood samples by centrifugation at 10,000 rpm for 10 minutes. After centrifugation, plasma layer gets separated and it is collected and stored at -20^{0}C for further use.

100µl of plasma and 400µl of acetonitrile were taken into efendrop tubes and centrifuged at 10,000 rpm for 10 minutes. The supernatant is collected and spike the drug made up with solvent. The sample is scanned in the range of 200 – 400 nm and the λ_{max} is determined and the absorbance is measured.

METHOD VALIDATION

All analytical procedures were validated according to USFDA guidelines 'Bioanalytical Method Validation'

Following parameters were performed for method validation

1. Sensitivity (LLOQ)
2. Linearity
3. Accuracy
    - Drug substance
    - Recovery studies
4. Precision
    - Intraday precision
    - Inter day precision
5. Stability studies[4]

Linearity with plasma

Preparation of Quality control (QC) standards in plasma: Samples were prepared by spiking with appropriate amounts of drug into 90 µL of control plasma. From 1 mg/mL stock solution of drug 10, 20, 30, 40, 50, 60, 70µL samples were taken and volume was made up to 10 mL with acetonitrile. From each concentration 10 µL of sample was taken and spiked into 90 µL control plasma to get

1000, 2000, 3000, 4000, 5000, 6000,7000ng/mL and the absorbance of these concentrations was measured spectrophotometrically at 250 nm against blank plasma. The obtained results were reported in table no. 01 and spectra shown in fig n0. 02 and graph no. 01

Sensitivity (LLOQ): It is the lower limit of quantification (LLOQ). The lowest concentration of the calibration curve where the analyte respond was more than five times the blank response was selected as LLOQ. At this concentration absorbance was reproducible with defined accuracy and precision. The spectrum were shown in **fig no. 03**

Accuracy: Accuracy was determined by spiking with appropriate amount of drug into 90µL of control plasma to get 1000, 1500, 4000 and 6500 ng/ml. 1000ng/mL - LLOQ, 1500ng/mL – LQC, 4000 ng/mL – MQC and 6500ng/mL – HQC.

The three standard solutions each five times were measured spectrophotometrically. Recovery determined should be within ±15%. The obtained results were reported in **t**abl**e** no. 02

Recovery: Recovery was determined at LQC (1500 ng/mL), MQC (4000 ng/mL) and LQC (6500 ng/mL) levels. The obtained results were reported in table no. 03

Precision: Samples were prepared by spiking required amount of drug into 90µL of plasma to get 1000, 2000, 3000, 4000, 5000, 6000 and 7000ng/mL.1000ng/mL – LLOQ, 1500ng/mL – LQC, 4000ng/mL – MQC and 6500ng/mL – HQC.

Variations of results within the same day (intraday) and variation of results between days (inter day) were analyzed.

Repeatability: Repeatability assessment of an analytical method was performed by analyzing six replicates of single concentration that is 4000ng/mL. Absorbance of samples were recorded at 250nm.The % CV was calculated. The obtained results were reported in table no. 04

Intraday precision: The intra-assay precision of the proposed method was determined on samples of drug solutions at varying concentration levels (LLOQ, LQC, MQC and HQC) by analyzing five replicates of each sample as a batch in a single run on the same day. Absorbance of samples were recorded at 250nm.The % CV was calculated. The obtained results were reported in table no.05

Inter day precision: The inter-assay precision was determined by analyzing the same samples of intraday precision on the next day. Absorbance of samples were recorded at 250nm.The % CV was calculated. The obtained results were reported in table no. 06

Stability studies

Short term stability: Three aliquots of each of the low (1000ng/ml) and high concentrations (6500ng/ml) were thawed at room temperature for 24 hours and then analyzed. Absorbance of samples were recorded at 250nm.The % CV was calculated. The obtained results were reported in table no. 07

Freeze and thaw stability: Drug stability was determined after three freeze-thaw cycles. Three aliquots of each of higher (7000ng/ml) and lower concentration (1000ng/ml) were frozen for 24 hours and then thawed unassisted at room temperature. When thawed completely, the same samples were frozen for 24 hours under the same conditions as before. Similarly the freeze and thaw cycles were repeated twice more and then analyzed on the third cycle. Absorbances of samples were recorded at 250nm.The % CV was calculated. The obtained results were reported in table no. 08

Stock solution stability: The stability of stock solution of the drug was evaluated at room temperature for 6 hours. Absorbance of samples were recorded at 250nm.The % CV was calculated. The obtained results were reported in table no. 09

RESULTS AND DISCUSIION

Table no. 01 Linearity

Concentration (ng/ml)	Absorbance
1000	0.078
2000	0.129
3000	0.199
4000	0.257
5000	0.312
6000	0.349
7000	0.407

Table no. 02 Accuracy

S.No	Concentration (ng/ml)	Mean absorbance ratio* ± standard deviation	% CV
1	LLOQ (1000ng/ml)	0.054 ± 0.0005	0.92
2	LQC (1500ng/ml)	0.071 ± 0.005	7.04
3	MQC (4000ng/ml)	0.191± 0.005	2.61
4	HQC (6500ng/ml)	0.324 ± 0.001	0.308

Average of five determinations; Acceptance criteria: NMT 15%

Table no. 03 Recovery Studies

Amount of standard (ng/ml)	Amount of sample (ng/ml)	Total concentration (ng/ml)	Total concentration Found (ng/ml)	% recovery
4000	1500	5500	5137	93.4
4000	4000	8000	7852	98.15
4000	6500	10500	9958	94.83

Table no. 04 Repeatability

S.NO	Concentration (ng/ml)	Absorbance
1	4000	0.342
2	4000	0.344
3	4000	0.344
4	4000	0.345
5	4000	0.345
6	4000	0.344
Mean	0.344	
Standard deviation	0.00109	
% CV	0.316	

Acceptance criteria: NMT 15%

Table no. 05 Intra-day precision

S.No	Concentration (ng/ml)	Mean absorbance*		Mean absorbance ±standard deviation	% CV
		Morning	Afternoon		
1	LLOQ(1000ng/ml)	0.035	0.034	0.0345 ± 0.0007	2.02
2	LQC (1500ng/ml)	0.054	0.046	0.05 ± 0.0056	11.2
3	MQC (4000ng/ml)	0.157	0.134	0.14 ± 0.016	11.42
4	HQC (6500ng/ml)	0.261	0.236	0.248 ± 0.017	6.85

Average of five determinations; **Acceptance criteria: NMT 15%**

Table no. 06 Inter-day precision

S.No	Concentration (ng/ml)	Mean absorbance*		Mean absorbance± standard deviation	% CV
		Day 1	Day 2		
1	LLOQ(1000ng/ml)	0.054	0.047	0.0505 ± 0.0049	9.80
2	LQC (1500ng/ml)	0.078	0.071	0.074 ± 0.0049	6.64
3	MQC (4000ng/ml)	0.194	0.191	0.192 ± 0.0021	1.107
4	HQC (6500ng/ml)	0.326	0.322	0.324 ± 0.0028	0.87

Average of five determinations; Acceptance criteria: NMT 15%

Table no. 07 Short term stability data

S.No	Concentration(ng/ml)	Time (hr)	Absorbance	Mean absorbance ± standard deviation	%CV
1	LLOQ(1000ng/ml)	0	0.053	0.051 ± 0.0028	5.49
		24	0.049		
2	HQC (6500ng/ml)	0	0.407	0.403 ± 0.0056	1.38
		24	0.399		

Acceptance criteria: NMT 15%

Table no. 08 Freeze and thaw stability data

S.No	Concentration(ng/ml)	Time(hr)	Absorbance	Mean absorbance ± standard deviation	% CV
1	LLQC(1000ng/ml)	0	0.084	0.078 ± 0.0084	10.76
		72	0.072		
2	HQC (7000ng/ml)	0	0.335	0.331 ± 0.0056	1.69
		72	0.327		

Acceptance criteria: NMT 15%

Table no. 09 stock solution stability data

S.No	Concentration(ng/ml)	Time(hr)	Absorbance	Mean absorbance ± standard deviation	% CV
1	LLOQ (1000ng/ml)	0	0.086**0.30.0**	0.08 ± 0.0084	10.5
		6	0.074		
2	HQC (7000ng/ml)	0	0.448	0.429 ± 0.0268	6.24
		6	0.410		

Acceptance criteria: NMT 15%

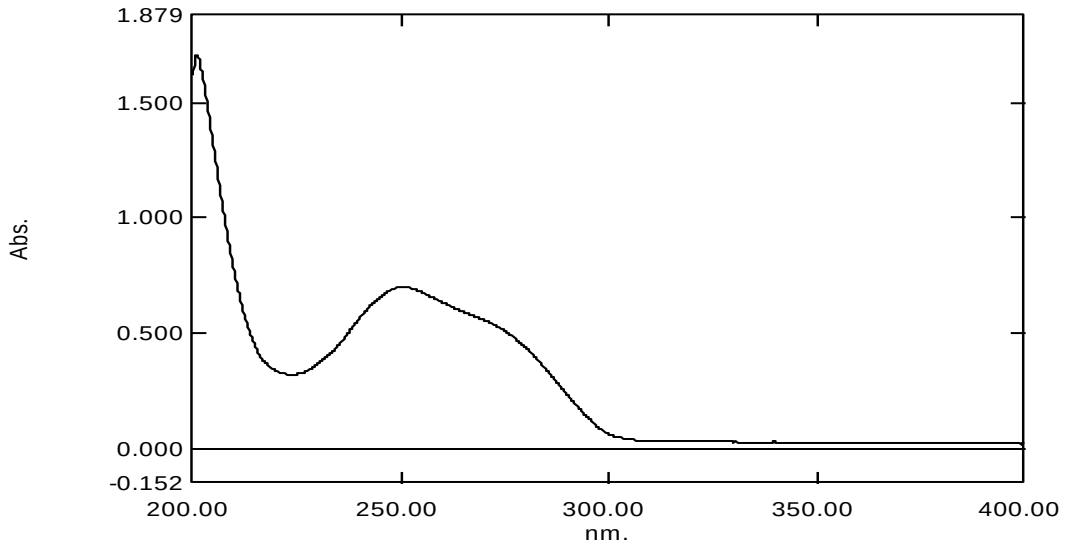


Fig no. 01 Absorption spectrum of rivaroxaban

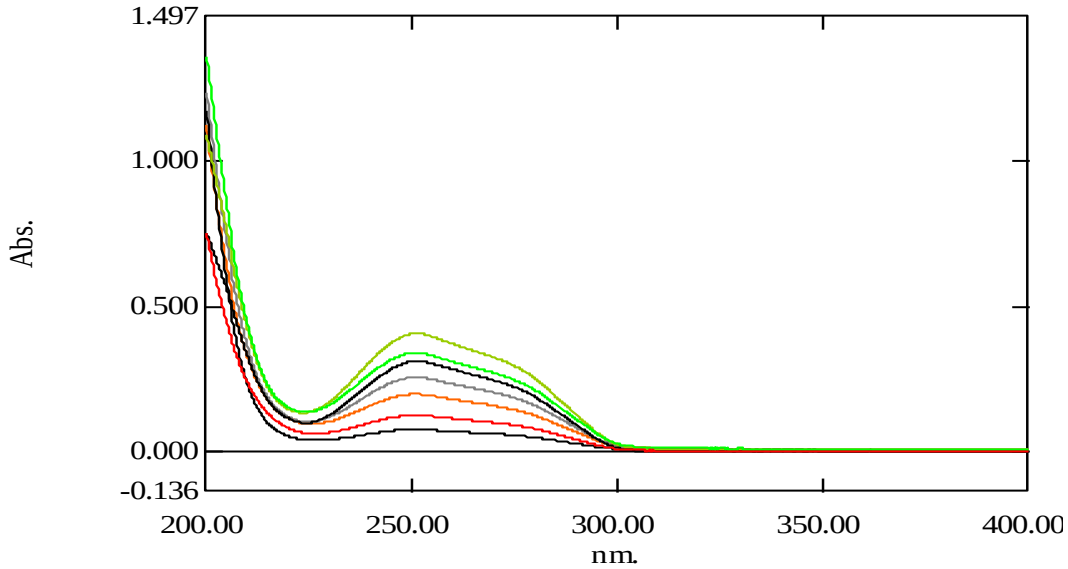


Fig no. 02 Overlay spectra of rivaroxaban (1000 – 7000ng/ml)

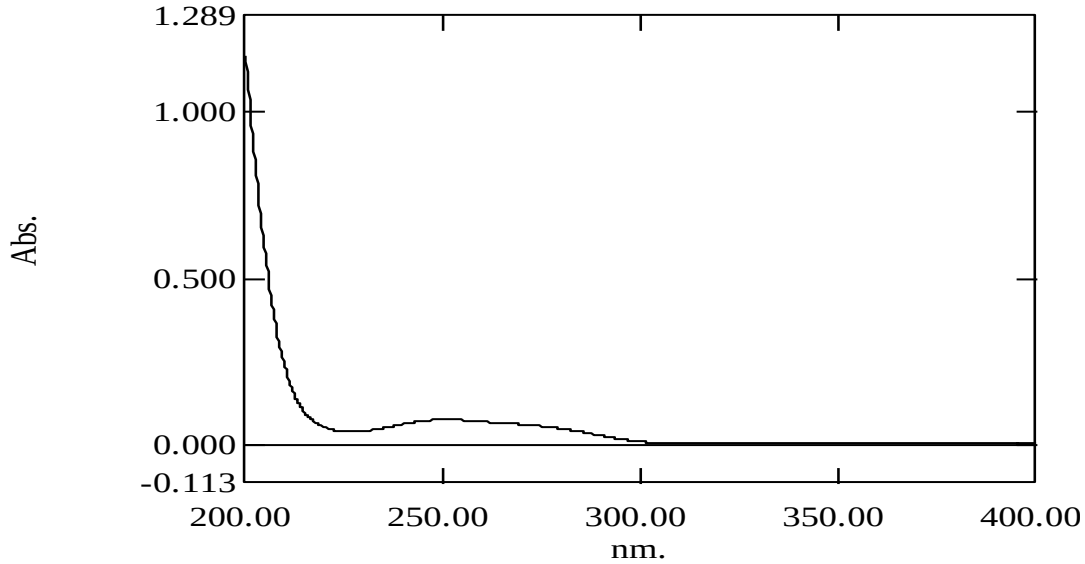
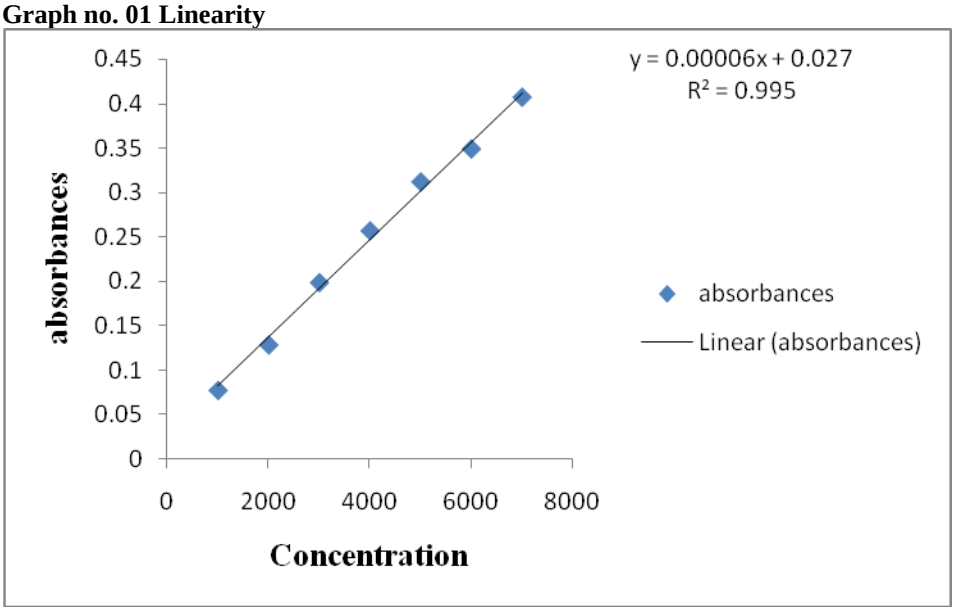


Fig no. 03 Spectrum of sensitivity (LLOQ

Graph no. 01 Linearity



CONLUSION

A new bioanalytical method for quantitative estimation of rivaroxaban using UV spectroscopic was developed and validated according to USFDA guidelines. All the parameters were found to be within the specified limits. From the results it concluded that the method is accurate and precise and it can be applied for regular analysis of rivaroxaban in spiked rat plasma.

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