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High performance liquid chromatography, TLC densitometry, first-derivative and first-derivative ratio spectrophotometry for determination of rivaroxaban and its alkaline degradates in bulk powder and its tablets

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ABSTRACT

Four sensitive and selective stability indicating methods for the determination of rivaroxaban (RIV) in the presence of its alkaline degradates (A,B) and in its pharmaceutical formulation were developed. Method A was an isocratic RP-HPLC,good resolution between peaks corresponding to the degradates from analyte was achieved on C18 column using mobile phase of 1.2% w/v potassium dihydrogen phosphate pH 3.5±0.2 and acetonitril (70:30,v/v). The detection was carried out at 280 nm. Method B depends on quantitative densitometric determination of thin layer chromatography (TLC) of rivaroxaban in the presence of its degradates without any interference.chloroform-isobutyl alcohol (50:50 v/v) was used as a developing system. The chromatogram was scanned at 280 nm. Method C was based on the first derivative (D^1) measurement of the drug at 237.4 nm,zero contribution point of its alkaline degradates.

Method D was based on the resolution of the drug and its alkaline degradates by first derivative ratio spectra (DD^1) and measured the amplitude at 236nm.These methods were successfully applied for the determination of rivaroxaban in bulk powder, pharmaceutical formulation and in presence of its alkaline degradates.The obtained results were statistically analyzed and compared with those obtained by the reported method.

KEYWORDS

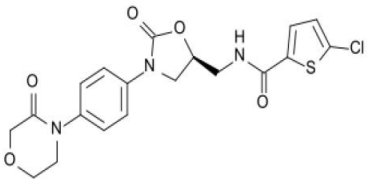
Rivaroxaban;
Its alkaline degradates;
RP-HPLC;
TLC;
First derivative;
First derivative ratio spectra.

INTRODUCTION

Rivaroxaban (RIV) is 5-chloro-N- ({ (5S) -2-oxo-3-[4- (3-oxomorpholin-4-yl) phenyl]-1,3-oxazolidin-5-yl}methyl) thiophene-2- carbinoxamide[1],as shown below

- Molecular formula: $C_{19}H_{18}ClN_3O_5S$,
- Molecular weight:435.9

Rivaroxaban (RIV) is an oral anticoagulant invented and manufactured by Bayer;it is marketed as Xarelto[2].It is



an oxazolidinone derivative anticoagulant that inhibit both free Factor Xa and Factor Xa bound in the prothrombinase complex[3]. It is a highly selective direct Factor Xa inhibitor with oral bioavailability and rapid onset of action. Inhibition of Factor Xa interrupts the intrinsic and extrinsic pathway of the blood coagulation cascade, inhibition both thrombin formation an development of thrombi. (RIV) does not inhibit thrombin (activated Factor II) ,and no effect on platelets have been demonstrated[2]. Few methods have been reported for the determination of (RIV) in human plasma including HPLC-tandem mass spectrometry (HPLC-MS/MS)[4,5] and HPLC in pharmaceutical formulation[6]. The novelty of research work was to develop a stability-indicating methods for the determination of (RIV) in presence of its alkaline degradates and the developed methods were validated with respect to specificity, LOD, LOQ[7], linearity, precision, accuracy and forced degradation study was performed on the drug substance to show the stability-indicating nature of the methods and also to ensure the compliance in accordance with International Conference of Harmonization (ICH) guidelines[8].

EXPERIMENTAL

Instruments

1 HPLC Agilant 1200 series was equipped with a model series ED4567677LC quaternary pump, Rheodyne injector Lot ID 7725 with a 20µL loop and UV detector DE71360313. Separation and quantitation were made on column phenomenex-C18,4.6×150mm,5µm particle size serial number:USK367776-4 (Germany) .

2 The detector was set at λ = 280 nm. The instrument was connected to an IBM compatible PC and HP disk jet printer.

3 -Densitometer-dual wave length SHIMADZU flying CS-9301. The instrumental parameter were: λ at 280nm,photomode:reflection,scan mode:zigzag and swing width:16.

4 A double beam UV-visible spectrophotometer (Shimadzu,Japan) model UV- 1601 PC with quartz cell of 1cm pathlength, connected to IBM

5 compatible computer and a HP 600 inject printer was used. The spectral band width was 2 nm and the wavelength scanning speed was 280nm.min^{-1}. The absorbence spectra of the test and reference solution were recorded over the range of 200-400 nm. The first derivative and derivative ratio were obtained using the accompanying software with

6 "λ = 4 and scaling factor = 10.

7 TLC plates Pre-coated with silica Gel GF_{254},0.25 mm thickness, (E.Merck Darmstadat,Germany) .

8 UV lamp-short wave length 254nm.

9 Mass spectrometer Jeol JMS –Ax500 Shimatzu, (Japan) .

10 FTIR spectrometer analytical,Nicolet 6700 (Thermo fisher scientific) .

11 pH meter HANAA 8417 (Portugal) .

12 Sonicator,Bandelin-Sonorex TK (Germany) .

13 Centrifuge Hettich (Germany) .

Materials and reagents

Pure standard

Rivaroxaban was kindly supplied by BAYER,Cairo Egypt;its purity was 99,08% ± 1.1 according to the reported HPLC method[6].

Pharmaceutical dosage form

Xarelto tablets®are labeled to contain 10 mg of rivaroxaban per tablet and manufactured by Bayer, Cairo, Egypt. Batch No. BXFT1R1.

Reagents and chemicals-

- All reagents and chemicals used were of analytical grade, and the solvents were of HPLC and spectroscopic grade.
- HPLC grade of Acetonitril & Methanol (SDFCL)
- Isobutyl alcohol,chloroform (Fisher Scientific) .
- Orthophosphoric acid, sodium hydroxide (Adwic).
- Hydrochloric acid (Riedel–de Häen) .
- Potassium di hydrogen orthophosphate (Winlab).
- The water for HPLC was prepared by double glass distillation and filteraion through a 0.45µm member filter.

Chromatographic condition

(a) For HPLC method

Analysis was carried out on Agilent 1200 series instrument. the elution was monitored at 280 nm. Separation was achieved by using column phenomenex (4.6×150mm,5µm) with mobile phase of (70:30,v/v)

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mixture of 1.2%w/v potassium dihydrogen orthophosphate pH 3.5±0.2 adjusted with orthophosphoric acid if necessary and acetonitril. The mobile phase was filtered using 0.45µm disposable filter (Millipore, Milford,MA) and degassed by ultrasonic vibration prior to use. The flow rate of the mobile phase was 1.5mL min^{-1}. Twenty microliters of sample solution was injected.

(b) For TLC method

The TLC plates were developed in chloroform: isobutyl alcohol (**50:50 v/v**) as a developing system. For detection and quantitation,5µL of different concentration of standard solutions within the quantitation range were applied as separate compact spots 15mm. apart and 20mm.from the bottom of the plate using 25 µL Hamilton analytical syringe.The plate was developed 16cm. in usual ascending way. The chromatographic jar was saturated with the developing system for 1hour. After elution, the plate was air dried and scanned at 280nm.as under the described instrumental parameters.

Preparation of alkaline degradates

The degradates were laboratory prepared according to the following method. In 100 mL stopper conical flask,25 mL of 2M aqueous sodium hydroxide solution was added to 50 mg of (RIV) and refluxed for 10 hours. The solution was cooled at room temperature then neutralized with 2M hydrochloric acid filtered if necessary,concentrated to 2 mls, transferred to 10 mL volumetric flask and diluted to the volume with acetonitril. The solution was tested for complete degradation by TLC using the chromatographic condition described above. The band corresponding to the degradation products were visualized under UV light at 254nm, scratched and extracted with acetonitril. The extracts were filtered and evaporated to dryness on a boiling water bath. The residue left after evaporation was used to prepare (1.0mg) in acetonitril stock degradation solution. Further dilutions were carried out with the same solvent for the laboratory prepared mixtures solutions and as in the proposed methods for (HPLC,TLC,D^1 and DD^1). The structure of the degradates was elucidated using IR and mass spectroscopy methods.

Stock standard solutions

(all stock standard solutions are freshly prepared)

- 1.0 mg mL^{-1} in acetonitril for HPLC and TLC methods.
- 400.0µg mL^{-1} in acetonitril for D^1 and DD^1.

Working standard solutions

- 200.00µg mL^{-1} in mobile phase for HPLC methods.
- 64.0µg mL^{-1} in acetonitril for D^1 and DD^1 methods.

Laboratory prepared mixtures

From both standard solutions,aliquots of (RIV) and its alkaline degradates, were accurately transferred into series of 10 mL volumetric flaskes,to prepare different mixtures containing (10-90%) of the alkaline degradates and the volume of each flask was completed with the same solvent used in the proposed methods.

PROCEDURE

Construction of calibration curve

(A) HPLC method

Aliquotes of (RIV) working standard solution (200.0µg mL^{-1}) in the mobile phase equivalent to (100.0-1200.0µg) accurately transferred into a series of 10 mL volumetric flaskes, the volume was completed to the mark with the mobile phase. Triplicate 20 µL injection were made for each concentration and chromatographed under the condition described above. The peak area of each concentration was plotted against the corresponding concentration to construct the calibration curve and then the regression equation was computed.

(B) TLC method

Aliquotes of (RIV) stock standard solution (1.0mg mL^{-1}) in acetonitril equivalent to (0.5-4.5mg) were accurately transferred into a series of 5mL volumetric flaske and the volume was completed to the mark with acetonitril. 5µL of each solution was applied to TLC plate (20cm×20cm) as separated spots. Triplicate applications were made for each solution and the plate was chromatographed as mentioned above. The spots were scanned with the spectrodensitometer at 280nm. The peak area of each concentration was plotted against the corresponding concentration to construct the

calibration curve and then the regression equation was computed.

(C) Spectrophotometric method

(a) For (D^1)

Aliquotes of (RIV) working standard solution (64.0µg mL^{-1}) in acetonitril equivalent to (16.0-224.0 µg) were accurately transferred into a series of 10 mL volumetric flaskes and the volume was completed to the mark with acetonitril. The amplitude of (D^1) values using acetonitril as a blank were measured at 237.4nm. The calibration curve was constructed then the regression equation was computed.

(b) For (DD^1)

Aliquotes of (RIV) working standard solution (64.0µg mL^{-1}) in acetonitril equivalent to (16-224 µg) were accurately transferred into a series of 10 mL volumetric flaskes, the volume was completed to the mark with acetonitril. Spectra of these solutions were divided by the spectrum of (20.0µg mL^{-1}) in acetonitril as a divisor of alkaline degradates. The (DD^1) values were calculated at 236nm for the obtained spectra with "λ=4 and a scaling factor =10. The calibration curve was constructed then the regression equation was computed.

Pharmaceutical formulation preparation:

Ten tablets of Xarelto® tablets were accurately weighed and finely powdered. A portion of the powder equivalent to 50.0mg of (RIV) was transferred into 50 mL volumetric flask,dissolved in 30 mL acetonitril with the aid of ultrasonic bath for 20 minutes. A sufficient quantity of acetonitril was added to produce 50mL. The solution was centrifuged for 20 minutes at 3500 rpm and then filtered;the final concentration (1.0mg mL^{-1}) . Further dilutions of the sample solution were carried out with the same solvent as in the proposed methods for HPLC and (D^1,DD^1) methods. The general procedures were completed as mentioned above, then,the concentrations of (RIV) was calculated from the corresponding regression equations.

Analysis of laboratory prepared mixtures

Each solution was analyzed as under the proposed methods and the concentration of the intact (RIV) was calculated from the corresponding regression equations.

RESULTS AND DISCUSSION

The advantage of the proposed methods is to be an stability- indicating methods which complete degradation and separation of the degradates with their identification with IR and Mass spectra were done.

The International Conference of Harmonization (ICH) guideline entitiled" stability testing of new drug substances and products" requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance[8]. An ideal stability indicating method is one that quantifies the standard drug alone and also resolves its degradation products. (RIV) was subjected to alkaline hydrolysis with 2M sodium hydroxide,complete degradation was observed and this is confirmed by TLC using (20cm×20cm) aluminium plates GF_{254} and chloroform – isobutyl alcohol (50:50 v/v) was used as a developing system. The R_F values of the intact (RIV) and its degradates (A,B) were found to be 0.60±0.02, 0.86±0.05 and 0.35±0.01 respectively. The band of each degradates was scratched and extracted with acetonitril. The extracts were filtered and evaporated to dryness on a boiling water bath. The residue left after evaporation were used for illucidation by IR and mass" spectroscopy.The structure assigned for one of the degradates (A) was confirmed through the formation of violet color with ninhydrin reagent, where a violet color was produced, indicating the presence of a free amino group. The IR spectrum of the degradate (A) (KBr) showed the appearance of broad band at 3453 cm^{-1} corresponding to the primary amino group and the degradate (B) showed the appearance of a band at 1735 cm^{-1} corresponding to the carbonyl group (Figure 1) . Also the structures of the degradates were confirmed by mass" spectroscopy (with up to n=3) . In degradate (A) the m/z value was 251 and in degradate (B) the m/z value was 144 (Figure 2) . Therefore one conclude that the alkaline hydrolysis of (RIV) may proceed as shown below

HPLC method

Several trials had been carried out to obtain a good resolution of the drug and its alkaline degradates. These trials involved the use of different mobile phases with different ratios,different pH and flow rates. The best

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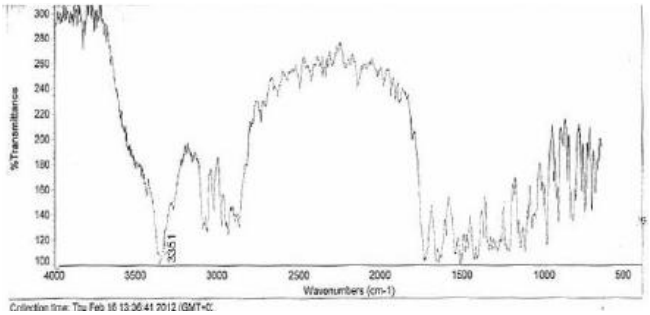


Figure 1(a) : IR spectra of (RIV)

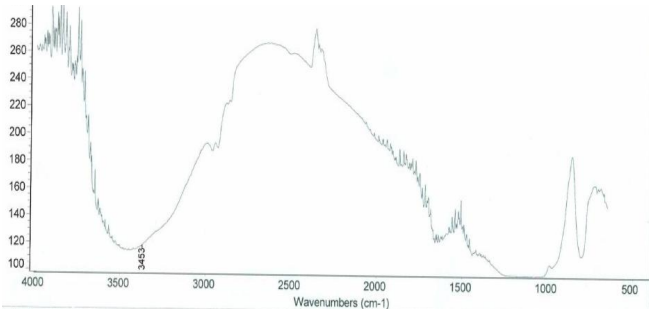


Figure 1(b) : IR spectra of degradation A

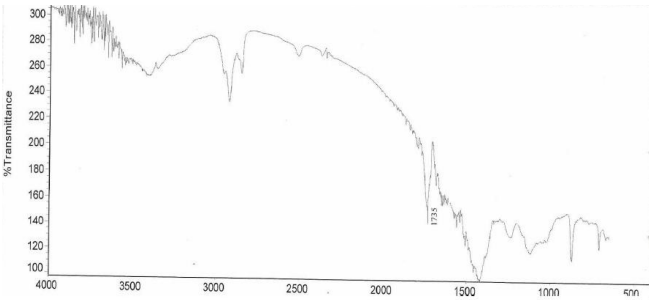


Figure 1(c) : IR spectra of degradation B

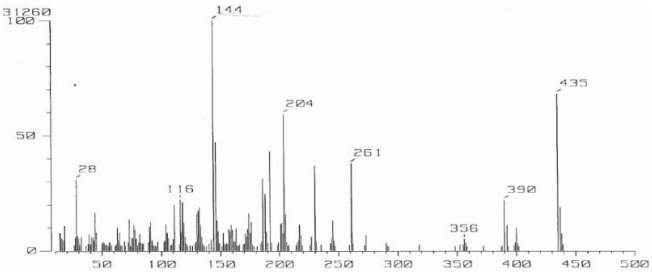
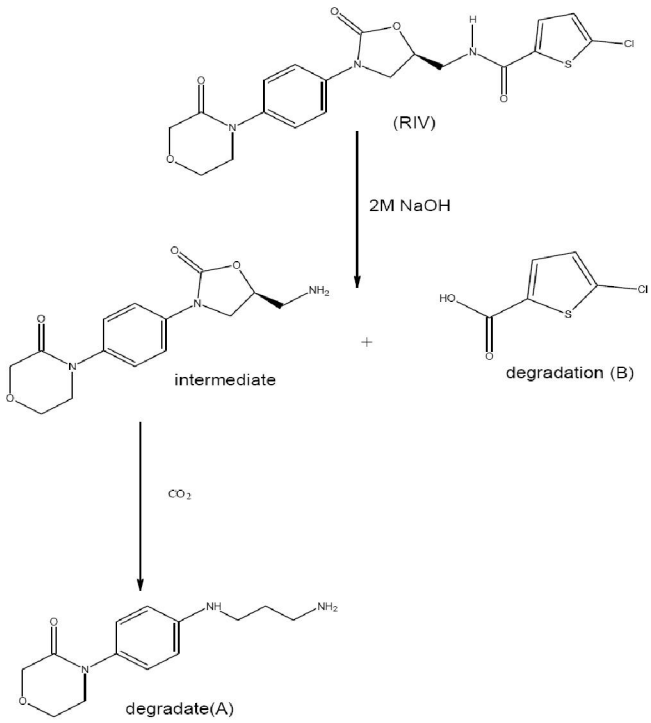


Figure 2(a) : Mass spectra of (RIV)

resolution with sharp and symmetric peaks were obtained upon using mobile phase of (70:30, v/v) mixture of 1.2% w/v potassium dihydrogen orthophosphate pH 3.5±0.2 adjusted with orthophosphoric acid and acetonitril The flow rate was 1.5mL min^{-1}. The retension time of (RIV) , degradates A and B were found to be 8.5±0.01, 5.3±0.01, 4.2±0.02, respectively as shown in (Figure 3). System suitability of the procedure was



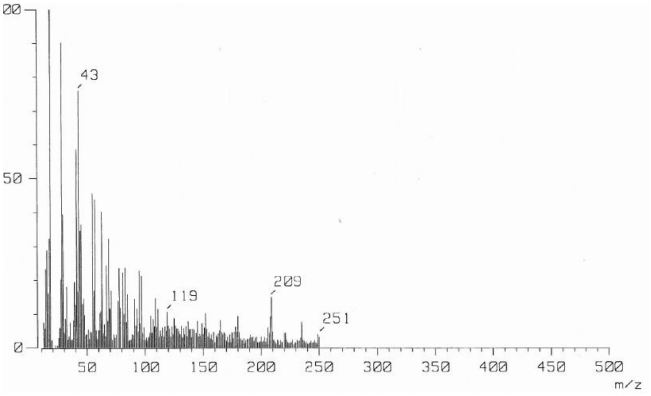


Figure 2(b) : Mass spectra of degradation A

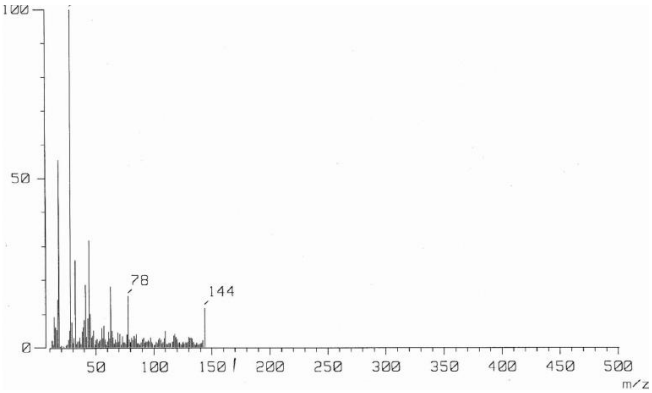


Figure 2(c) : Mass spectra of degradation B

shown in (TABLE 1) . Characteristic parameters for regression equation and correlation coefficient obtained by least square treatment of the results were given in

TABLE 1 : System suitability parameters of the proposed HPLC method

Parameter	Rivaroxaban	Degradation A	Degradation B
Resolution	4.85	6.06	3.25
Tailing factor	1.2	125	1254
Retention time (min.)	8.5±0.01	5.3±0.01	4.2±0.02
Symmetry	0.86	0.95	0.64
Column efficiency (N)	14886	10943	11999

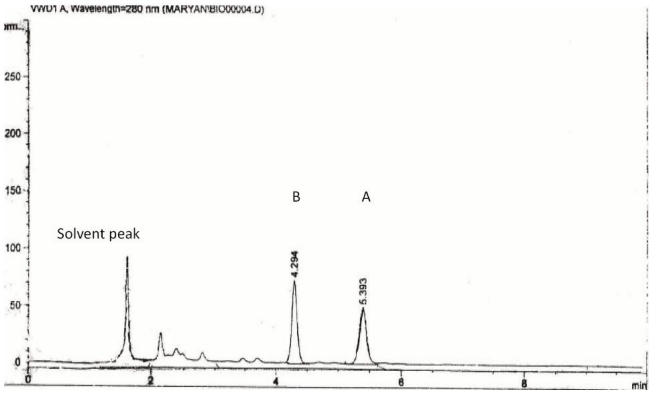


Figure 3 (a) : HPLC Chromatogram of mixture of alkaline degradates(A&B) 10µg mL^{-1} of each in mobile phase showing complete degradation.

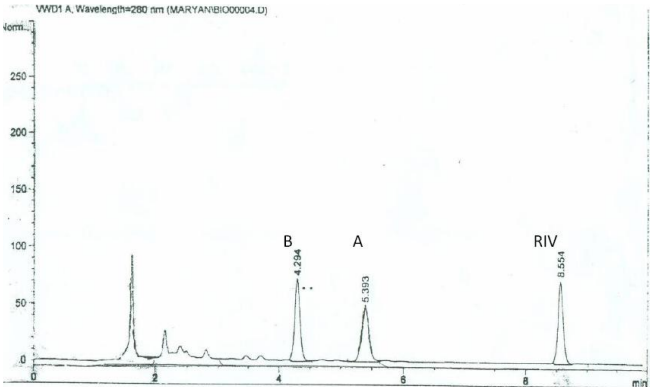


Figure 3(c) : HPLC Chromatogram of mixture of (RIV) 10µg mL^{-1} and its alkaline degradates(A&B) 10µg mL^{-1} of each in mobile phase

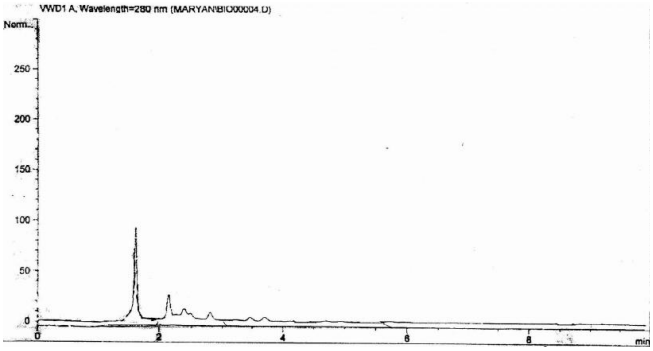


Figure 3(b) : HPLC Chromatogram of mobile phase

(TABLE 2) and the corresponding concentration in the range10-120µg mL^{-1}, from which the regression equation was computed as shown in (TABLE 2) .

TLC method

The experimental conditions for TLC method as developing system,scan mode and wavelength of the detection were optimized to provide accurate and precise results for the determination of (RIV) in the presence of its degradates. The chosen scan mode with zigzag and the wavelength of scanning was 280 nm.

TABLE 2 : Characteristic parameters of rivaroxaban by the proposed methods

Characteristic parameters	HPLC	TLC	Derivative Spectrophotometry	Ratio derivative
Linearity and range µg mL^{-1}	10.0-120.0	0.5- 4.5 ?	1.6-22.4	1.6-22.4
Mean ±%RSD (accuracy)	101.0±1.6	100.5.±1.5	99.6±1.0	100.9±0.7
Correlation coefficient (r)	0.9997	0.9999	0.9989	0.9997
Regression equation				
Slope	40.124	1786.3	0.0238	0.2502
RSD% of slope	0.02	1.1	0.004	0.01
Intercept	-4.4618	-53.517	0.01	0.008
RSD% of intercept	1.2	0.1	0.001	0.001
LOD ?, µg/ml	0.09	0.01	0.179	0.03
LOQ ? ?, µg/ml	0.3	0.04	0.599	1. 7
Intraday precision[a]	99.5±1.6	99.3±1.1	100.7±1.2	100. 8±1.2
Interday precision[b]	99.4±1.6	99.7±1.2	100.6±1.2	100.5

? **µg/spot; [a]The intraday (n=3), average of three different concentrations repeated three times within day; [b]The interday (n=3), average of three different concentrations repeated three times in three successive days.**

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smoothed with Δλ=4 intervals (Figure 6.a) and their first derivatives were traced with the same Δλ (Figure 6.b) . The concentration of (RIV) was determined by measuring the amplitude at λ max 234nm. The main instrumental parameter conditions were optimized for a reliable determination of (RIV). Some divisor concentrations were tested,which is very important factor in practice. The influence of the Δλ for first derivative ratio was tested and found very appropriate to use the values at Δλ=4. In this method the

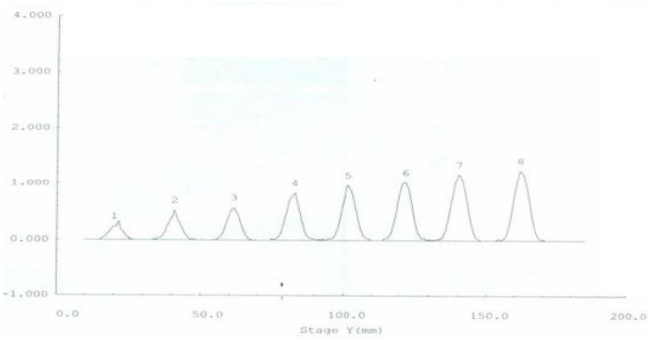

Figure 4 : High Performance Thin layer Densitogram of rivaroxaban in acetonitril (Rf =0.60) ,concentration range (0.5-4.5µ/spot) at 280 nm.using chloroform-isobutylalcohol(50:50 v/v) as a developing system

The best separation of the studied drug and its degradates were obtained by using chloroform-isobutylalcohol (50:50 v/v) as a developing system. R_F values for intact (RIV) , and its degradates (A,B) were found to be 0.60±0.02, 0.86±0.05 and 0.35±0.01, respectively. A linear correlation was obtained between the area under the peak and the concentration (0.5-4.mg/spot) as shown in Figure 4 and the regression equation was computed as shown in (TABLE 2) .

Spectrophotometric methods

First derivative (D^1)

The zero-order absorption spectra of (RIV) and its degradates showed sever overlapping (Figure 5.a) . For this reason the conventional UV spectrophotometry can not be used in this case. However, zero-crossing first-order spectrophotometry is more selective and permit identification and determination of (RIV) in the presence of its degradates. (Figure 5.b) shows the (D^1) of (RIV) zero-crossing of its degradates. The selection of the optimum wavelength is based on the fact that the absolute value of the total derivatives spectrum at the selected wavelength has the best linear response to the

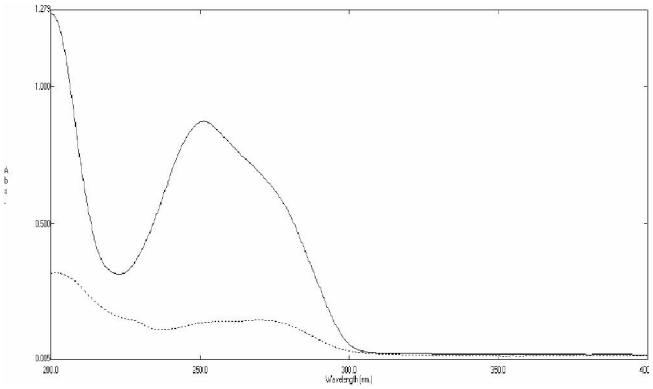

Figure 5(a) : Zero order absorption spectra of (RIV) 12.0µg mL^{-1} in acetonitril (_____) and its alkaline degradates 12.0 µg mL^{-1} in acetonitril (…….)

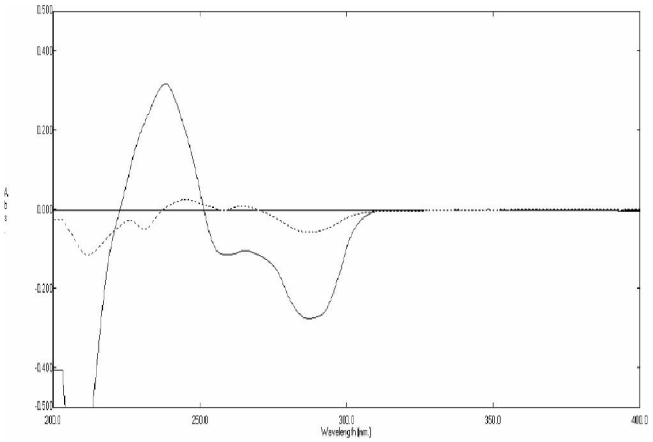

Figure 5(b) : First derivative spectra of (RIV) 12.0µg mL^{-1} in acetonitril (_____) and its alkaline degradates 12.0 0µg mL^{-1} in acetonitril (…….).

analyte concentration. It is not affected by the concentration of any other components and gives a near zero-intercept on the ordinate axis of the calibration curve. Therefore determination of (RIV) at 236nm (zero-crossing of its degradates) was chosen as optimum working wavelength. Beers law was obeyed in concentration range (1.6-22.4µg mL^{-1}) from which the linear regression equation was computed as shown in (TABLE 2) .

First derivative ratio spectra (DD^1)

Mixtures of compounds with highly overlapped spectra have been resolved by the use of the ratio spectra with high accuracy[9-11]. The absorption spectra of (RIV) prepared at different concentration were recorded in the range of 200-400nm and stored in IBMPC.The stored spectra were divided by a spectrum of degradates 20.0µg mL^{-1}. The ratio spectra were

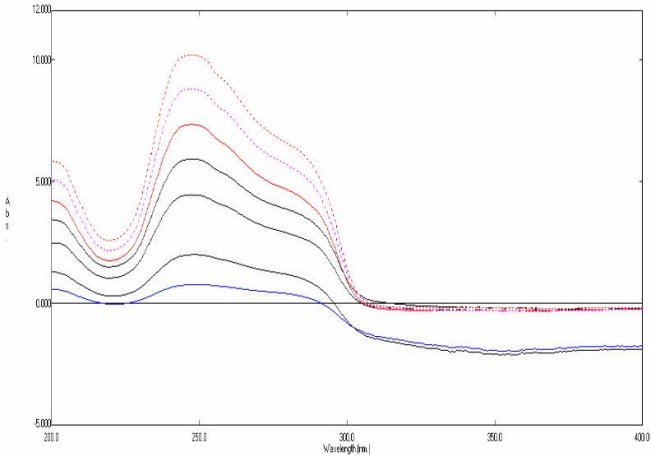

Figure 6(a) : Ratio spectra of RIV (1.6-22.4µg mL^{-1}) in acetonitril with the divisor of alkaline degradates 20.0 µg mL^{-1} in acetonitril.

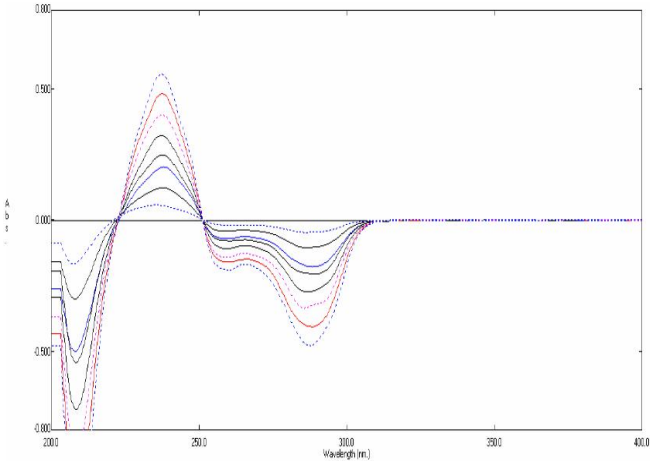

Figure 6(b): First derivative ratio spectra of RIV (1.6-22.4µg mL^{-1}) in acetonitril with the divisor of alkaline degradates 20.0 µg mL^{-1} in acetonitri

concentration range (1.6-22.4 µg mL^{-1}) was used from which the regression equation was computed as shown in (TABLE 2) .

The specificity of the proposed methods was assessed by the analysis of laboratory prepared mixtures, the results in (TABLE 3) ,indicating that the proposed methods were not affected by the presence of degradates up to 90% of the degradates. Methods validation was performed according to the ICH guidelines[8] for all the proposed methods. (TABLE 2) shows the results of linearity, accuracy, repeatability, precision and robustness of the proposed methods. The

TABLE 3 : Evaluation of the selectivity of the proposed methods for the dertermination of Rivaroxaban in the presence of its alkaline degradates

Samlpe number	% added of degradates	% recovary			
		HPLC	TLC	D^1	DD^1
1	10	100.2	98.4	99.1	101.1
2	20	99.8	100.7	101.9	98.5
3	30	98.2	99.7	100.3	101.6
4	40	101.2	98.9	101.1	100.9
5	70	100.6	101.4	100.9	99.3
6	80	99.5	99.3	100.2	101.5
7	90	101.5	101.7	99.1	100.8
Mean±%RSD			100.1±1.1	100.00±1.3	100.4±1.0

TABLE 4 : Determination of Rivaroxaban in pharmaceutical formulation by the proposed methods.

Parameter	HPLC	TLC	D^1	DD^1	Reported method[6]e
Mean±%RSD^a (Xarelto)^b	98.44±0.3	101.87±0.1	99.71±1.7	101.52±0.1	99.08±1.1
Variance	0.6	1.6	1.8	1.4	1.1
N	5	5	5	5	5
t- test (2.30)^c	0.11	0.10	0.82	0.15	_____
F-ratio (6.39)^c	1.85	1.45	1.63	1.23	_____
%Recovery ^{d}	100.8±1.1	99.7±0.3	100.20±0.5	99.74±1.1	_____

a-Percentage relative standard deviation for five determinations; b-Labeled to contain 10 mg per tablet; c-Theoretical value for t and F for P= 0.05; d-Standard addition of different concentrations of rivaroxaban; e-Reported HPLC method using C18(4.6×10 cm 5µm) column, mobile phase acetonitril and water pH; 3(30:70),flow rate 1.2mL min^{-1} and detection at 250 nm.The retension time of rivaroxaban was at 2.5min.(not stability indicating method).

Review

TABLE 5 : Statistical comparison between the proposed methods and the reported method in bulk powder

Parameter	HPLC	TLC	D^1	DD^1	Reported method[6]c
Mean ±%RSD[a]	101.0±1.6	100.5±1.5	99.6±1.0	100.9±0.7	99.08±1.1
Variance	2.7	2.2	1.1	0.5	1.5
n	7	7	7	7	7
t- test (2.02)[b]	0.1	0.2	1.1	1.2	
F-ratio(4.39)[b]	1.7	1.5	1.4	3	

a- Average of seven determinations; b- Values of theoretical t and F values at P=0.05; c- Reported HPLC method using C18(4.6×10 cm 5µm) column, mobile phase acetonitril and water pH 3(30:70), flow rate 1.2mL min^{-1} and detection at 250 nm. The retension time of rivaroxaban was at 2.5min. (not stability indicating method).

robustness of the method was evaluated by observing the influence of small variations of experimental variables, for example, the change of the pH of potassium dihydrogen orthophosphate pH 3.5±0.2. This minor change that may take place during the experimental operation did not affect the absorption intensity indicating the excellent robustness of the proposed method. The proposed methods were applied to determine of (RIV) in the commercial tablets. Five replicate determinations were done and satisfactory results were obtained in a good agreement with the tablets which labeled to contain 10 mg per tablet as shown in (TABLE 4) . The validity of the proposed methods was assessed by applying the standard addition technique, which showed accurate results and there is no interference from excipients as shown in (TABLE 4) . Statistical comparison of the results of bulk powder obtained by the proposed methods and the reported HPLC method[6] was also done using student' s t-test and F-ratio at 95% confidence level as in (TABLE 5) . It is clear that there is no significant difference between the proposed methods and the reported one with regard to accuracy and precision.

RESULTS AND DISCUSSION

In this paper a sensitive,specific,accurate,precise validated and well defined stability indicating HPLC, TLC, (D^1) and (DD^1) methods were investigated. The chromatographic methods HPLC and TLC were found to be more sensitive than the spectr ophotometric methods. The HPLC method was found to be more sensitive than the TLC method, while the later has the advantages of short run time, large sample capacity and the use of minimal volume of solvent. The (D^1) and (DD^1) methods have the advantages of low cost,rapid and environmental protection. The proposed methods were suitable for quality control laboratories,where economy and time are essential.

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