

Spectrophotometric quantification of direct factor xa inhibitor, rivaroxaban, in raw and tablet dosage form

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Abstract

Two simple spectrophotometric methods (I and II) are described for the determination of rivaroxaban in bulk and tablets. Method I is based on the reaction of rivaroxaban with 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole in alkaline medium. Method II involved the reaction of rivaroxaban with *p*-Dimethylaminocinnamaldehyde in acidic medium. The reactions products were measured at 405 nm and 545 nm for methods I and II, respectively. Beer's law was obeyed over the concentration ranges of 2-20 µg/mL and 25-125 µg/mL with lower limits of detection of 0.110 µg/mL and 0.483 µg/mL for Methods I and II, respectively. Percent recovery of rivaroxaban for both methods I and II were found in the range of 99.74-100.51% with relative standard deviation in the range of 0.621-0.900%. The proposed methods I and II were successfully applied for quantification of rivaroxaban in tablet dosage forms with good accuracy and precision. Hence, the proposed methods I and II can be suitably adopted for routine analysis of rivaroxaban.

Introduction

Rivaroxaban (RXN), chemically also known as (S)-5-Chlor-N-{2-oxo-3-[4-(3-oxomorpholin-4-yl) phenyl]-1,3-oxazolidin-5-ylmethyl}thiophen-2-carbamide. RXN is a novel, oral, direct Factor Xa inhibitor approved for the management of deep vein thrombosis and pulmonary embolism [1-3]. RXN is also employed to cut down the danger of forming a blood clot in the legs and lungs of adult patients who have undergone hip or knee replacement surgery [4,5]. RXN is a direct Factor Xa inhibitor, which binds directly to free and clot bound factor Xa. After binding, it effectively blocks the amplification of the coagulation cascade, preventing the formation of thrombus [6].

A variety of analytical methods have been proposed for the estimation of RXN in bulk, pharmaceutical dosage form and biological fluids. They include factor Xa specific chromogenic substrate assay [7], anti-Factor Xa chromogenic assay [8-10], prothrombin time assay [11], HPLC-MS/MS [12-14], HPTLC [15,16], HPLC [16-26] and UPLC [27]. The above reported methods suffer from drawbacks such as time consuming, cumbersome procedure, costly and require an expertise operational personal [7-27]. Some of the methods are applicable only for the plasma samples [7-14].

Spectrophotometry is an analytical technique for the enhancement of sensitivity, simplicity, cost effectiveness and specificity in quantitative analysis of a variety of pharmaceutical compounds. In the existing literature, there are few reports regarding the use of spectrophotometry for the quantification of RXN in bulk and tablet dosage forms. Determination of RXN in the presence of its alkaline degradation products by two different approaches is proposed by Lories *et al.* [16]. The first method is the zero-crossing first-order spectrophotometry technique, in which 236 nm is chosen as λ_{\max} for the determination of RXN. The second method is the first derivative ratio spectra in which the absorption spectra of RXN with different concentrations were recorded in the range of 200-400 nm and the spectra obtained were divided by a spectrum of alkaline degradates. The ratio spectra were

smoothed with $\Delta\lambda=4$ intervals and their first derivatives were traced with the same $\Delta\lambda$. The concentration of RXN was determined by measuring the amplitude at λ_{\max} 234 nm.

Muralikrishna & Kasad [28] and Sekaran *et al.*, [29] described UV spectrophotometric methods for the quantification of RXN. Measurement of the absorbance of methanolic solution of RXN at 248.6 nm and dimethyl sulphoxide solution of RXN at 270 nm has served as the basis for the determination of RXN in the methods of Muralikrishna & Kasad [28] and Sekaran *et al.*, [29], respectively. The area under curve spectrophotometry technique proposed by Kasad & Muralikrishna [30] involves the calculation of integrated value of absorbance of RXN in methanol solution with respect to the wave length between two selected wave lengths 241 nm and 260 nm. The UV spectrophotometric methods [16,28-30] are simple but they suffer from lack of selectivity as they involve measurements at shorter wavelength. Sekaran *et al.*, [29] method is not applied to tablet dosage forms.

Satyanarayana & Madhavi have proposed five visible spectrophotometric methods (A-E) for the quantification of RXN in formulations [31]. The reaction schemes involved in the five methods are: oxidation of RXN with ferric chloride and the estimation of Fe (II) produced after chelation with 2,2'-bipyridyl (method A); Schiff's base formation of the RXN with 4-amino phenazone (method B); Charge transfer complexation of RXN with haematin formed from the reaction between haematoxyline and chloramine T in basic media (method C);

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Condensation of RXN with isonicotinic hydrazide (method D); and Condensation of RXN with 1,2-naphthaquinone-4-sulfonic acid sodium in alkaline media (method E). The Satyanarayana & Madhavi [31] methods suffers from one or more disadvantages like less sensitive, lack of accuracy & precision and requirement of extraction procedure. The Satyanarayana & Madhavi [31] methods were not fully validated according to ICH guidelines. The reaction optimization details, method validation parameters such as selectivity, robustness and ruggedness were not reported in the methods of Satyanarayana & Madhavi [31].

As an electroactive halide reagent, 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD) was used as an analytical reagent for the estimation of a number of amines and amino acids [32]. NBD has been used as a fluorogenic or chromogenic reagent in pharmaceutical analysis. The analysis of different compounds/drugs either in pharmaceuticals/biological samples was performed after derivatization with NBD followed by measuring the resulted product by means of spectrophotometry [33-38] and spectrofluorimetry [39,40]. Due to electrophilic properties, NBD has also been used in charge transfer reactions. This property is used in the quantification of various β -blockers [41], skeletal muscle relaxant [33] and antihistaminic drugs [33].

Under the appropriate conditions, enamines are formed by the condensation of a secondary amine with an aldehyde or ketone in the presence of an acid catalyst [42,43]. The formation of enamine forms the basis for the quantification of compounds of pharmaceutical significance using spectrophotometry [44-46]. The condensation of *p*-dimethylaminocinnamaldehyde (PDAC), also referred as Renz and Loew reagent [47], with compounds having secondary amine to form enamines has been applied for their quantification using spectrophotometry [48,49].

In the present study, the authors investigated the reaction of RXN with NBD (method I) & PDAC (method II) and employed these reactions in the development of two new spectrophotometric methods for the determination of RXN in bulk and tablets. The developed methods were fully validated following the ICH guide lines. The performance of the reported and proposed spectrophotometric methods is tabulated in Table 1.

Materials and methods

Instrumentation

- ELICO (Hyderabad, India) double beam model SL 159 digital

spectrophotometer with one cm matched quartz cells.

- Kemi (Ernakulam, India) KWB 220 model water bath.
- Essae-Teraoka electronic weighing balance (Goa, India) PG1000 model.
- Elico (Hyderabad, India) LI120 model pH meter.

Reagents: All chemicals were of analytical reagent grade. All the solutions were prepared afresh daily. 0.1% (w/v) NBD (Merck, Mumbai, India) in methanol (Merck, Mumbai, India) and 0.2 M Borate buffer (pH-7.8) were prepared and used in method I. 3% (w/v) PDAC (Sd fine Chem Ltd., Mumbai, India) in methanol and sulphuric acid (Sd fine Chem Ltd., Mumbai, India) were used in method II.

Standard solutions: Pharmaceutical grade RXN was obtained as gifted sample from MSN laboratories, Hyderabad, India. RXN was used as received. Tablet dosage forms of RXN (Xeralto tablets, Bayer India Limited Mumbai, India, labeled to contain 10 mg of RXN/tablet) were employed in the present investigation. A stock standard solution containing 1 mg/mL of RXN was prepared in methanol. Working standard solution equivalent to 100 μ g/mL (method I) and 500 μ g/mL (method II) of RXN were obtained by appropriate dilution of stock solution with methanol.

General analytical method

Method I: Into a series of boiling test tubes, different volumes (0.2-2.0 mL) of RXN (100 μ g/mL) were pipetted. To each test tube, 3 mL of borate buffer and 1.0 mL of 0.1% NBD were added, mixed well, and heated on a water bath at 60°C for 25 min. The tubes were cooled at room temperature, and then the contents of the tubes were transferred to 10 mL volumetric flasks. To each flask 0.2 mL of HCl was added and diluted to volume with methanol. The absorbance of each solution was measured at 405 nm against a reagent blank treated similarly except without drug.

Method II: Into a series of 10 mL volumetric flasks, different volumes (0.5-2.5 mL) of RXN (500 μ g/mL) were pipetted. To each test tube, 2 mL of 3% PDAC and 1.0 mL of H₂SO₄ were added, mixed well, and kept aside for 10 min at room temperature. The contents of the flasks were diluted to volume with methanol. The absorbance of each solution was measured at 545 nm against a reagent blank treated similarly except without drug.

Construction of calibration curve: For both the proposed

Table 1. Performance of proposed and reported spectrophotometric methods for the assay of RXN.

Sl no.	Reagent/ solvent	λ max (nm)	linearity (μ g/mL)	LOD & LOQ (μ g/mL)	RSD (%)	Recovery (%)	Reference
1	ACN	237.4	16-224	0.52 & 1.86	1.329	99.52	16
2	ACN	amplitude at 234	16-224	0.62 & 0.65	0.639	100.84	
3	MeOH	248.6	2-12	0.09842 & 0.2982	0.023-0.915	100.85	28
4	DMSO	270	2-20	0.212 & 0.642	0.01- 0.50	99.82-100.50	29
5	MeOH	241 & 260	2-12	0.059 & 0.179	0.297-0.537	99.31	30
6	BPD	470	2-20	0.03 & 0.1	0.47	98.17	31
7	AP	450	3-21	0.15 & 0.5	0.88	98.58	
8	HMN	740	30-90	9 & 10	0.94	98.53	
9	INH	470	5-30	0.15 & 0.5	0.52	99.30	
10	NQS	500	15-90	1.5 & 5	1.02	99.25	Proposed method I
11	NBD	405	2-20	0.110 & 0.333	0.252-0.487	99.75-100.20	
12	PDAC	545	25-125	0.483 & 1.463	0.408-0.692	99.74-100.50	Proposed method II

(ACN-Acetonitrile; MeOH-Methanol; DMSO-dimethyl sulphoxide; BPD-2,2-Bipyridine; AP-4-Amino phenazone; HMN- Haematoxylin; NQS-1,2-Naphthaquinone-4-sulphonate; 1,10-PTL-1,10-Phenanthroline; CH₃CHO/CRL-Acetaldehyde/Chloranil; NBD- 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole; PDAC- p-Dimethylaminocinnamaldehyde).

methods (A and B), the calibration curves were constructed by plotting the absorbance against the final concentration of the drug. The corresponding regression equations were derived. The concentration of the unknown samples were read from the corresponding calibration graph or computed from the corresponding regression equation.

Procedure for the analysis of RXN in tablet dosage forms: Twenty Xeralto tablets (Bayer India Limited Mumbai, India) claimed to contain 10 mg of RXN were weighed and pulverized. An amount of powder equivalent to 100 mg of RXN was weighed into a 100 ml volumetric flask, 50 mL of methanol was added and shaken thoroughly for about 10 min. The volume of the flask was diluted up to the mark with the same solvent, mixed well and filtered using a quantitative filter paper. The filtered solution was suitably diluted with the methanol. Convenient aliquots were subjected to analysis by the procedures described under methods I and II. The nominal content of RXN in the tablet was calculated from the corresponding calibration curve or regression equation.

Results and discussion

Basis of the color reaction

Method I: Novel spectrophotometric methods were developed through derivatization of the secondary amino group of the drug with NBD, which is known to react with primary and secondary amines forming stable condensation colored products. The method I is based on condensation of secondary amine in the RXN with NBD in the presence of alkaline borate buffer (pH 7.8). The colored complex has a characteristic absorption spectrum with a maximum absorption at 405 nm against the reagent blank (Figure 1). The proposed reaction scheme is presented in (Figure 2).

Method II: Primary and secondary amino groups present in the drug undergo condensation with carbonyl reagents (such as *p*-dimethylaminobenzaldehyde, *p*-dimethylamino cinnamaldehyde and vanillin) in acidic condition to form colored chromogen with characteristic absorption maxima. This reaction was utilized in the present investigation for the determination of RXN by the method II. The secondary amino group of RXN was found to react with PDAC in the presence of sulphuric acid as a catalyst resulting in formation of a red colored enamine complex. The colored complex has a characteristic absorption spectrum with a maximum absorption at 545 nm against the reagent blank (Figure 3). The probable reaction mechanism is given in (Figure 4).

Optimization of experimental conditions

The experimental conditions in method I were established by studying the effect of various parameters like volume and concentration of NDB, buffer pH, temperature and reaction time for the maximum

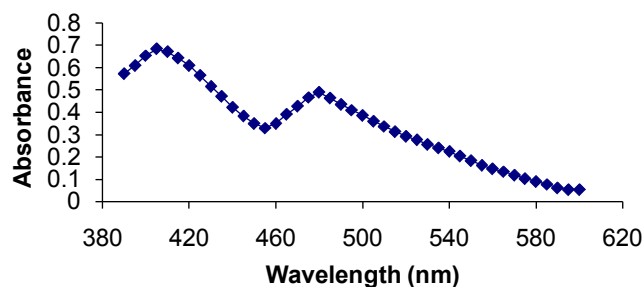


Figure 1. Absorption spectra of RXN-NBD complex.

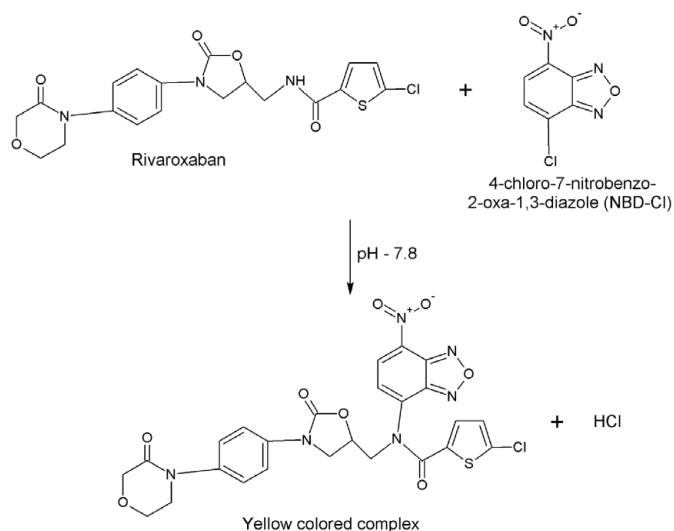


Figure 2. Condensation reaction between rivaroxaban and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole.

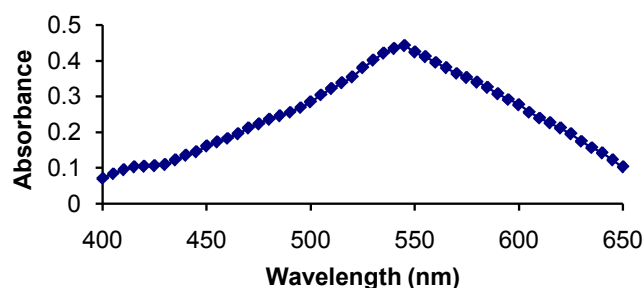


Figure 3. Absorption spectra of RXN-PDAC complex.

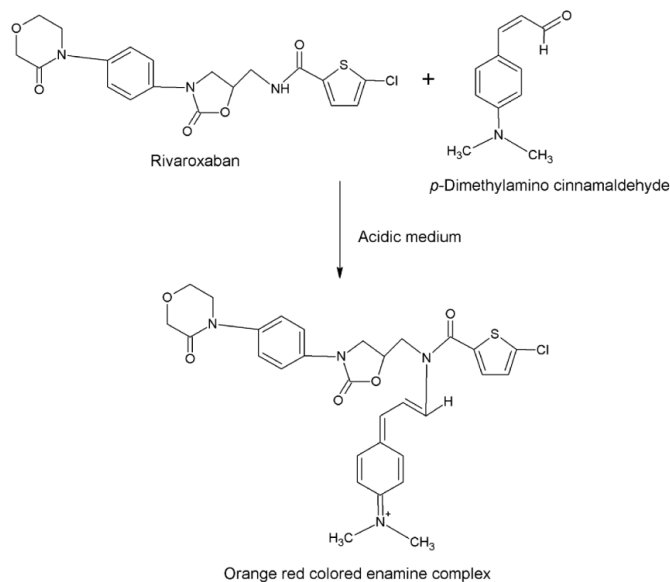


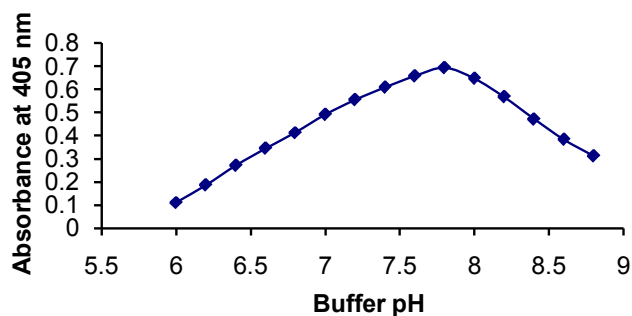
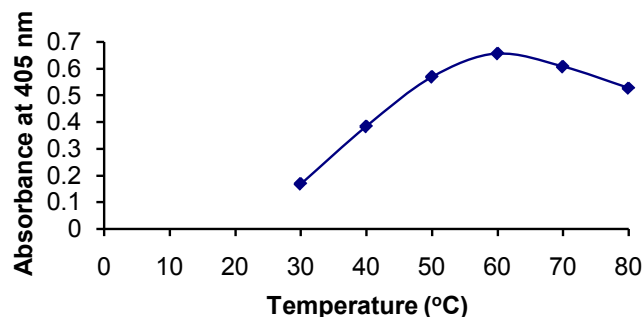
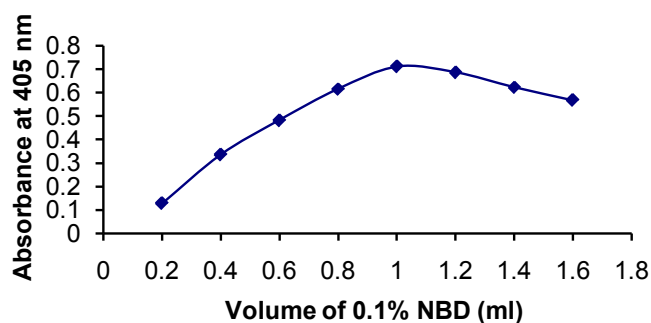
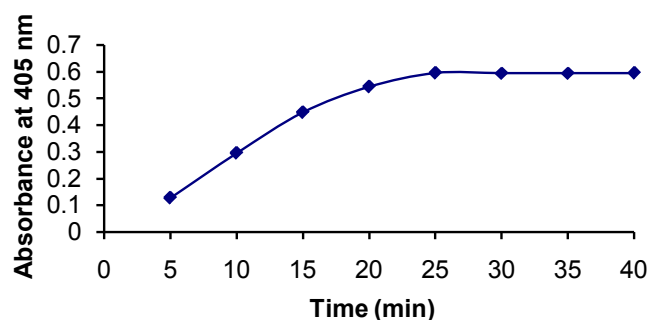
Figure 4. Enamine complex formation between rivaroxaban and *p*-dimethylamino cinnamaldehyde.

and stable color development. The results are incorporated in (Table 2) (Figure 5-8).

The experimental conditions in method II were established by studying the effect of various parameters like volume and concentration

Table 2. Optimization of conditions for the assay of RXN by Method I.

Parameter	Investigation conditions	Optimized condition	Remarks
Effect of pH of buffer	6.0-8.8	7.8	The absorbance increases in the media of pH from 6.0-7.8 and there is a decrease in the absorbance with the further raise in pH (Figure 5)
Volume of 0.1% NDB (mL)	0.2-1.6	1.0	One mL of NDB gave maximum absorbance. There is decrease in the absorbance with further increase in the volume of reagent (Figure 6)
Temperature (°C)	30-80	60	The absorbance increased with increasing temperature up to 60°C, and higher temperature had negative effect (Figure 7).
Effect of reaction time (min)	5 – 40	25	Maximum absorbance was obtained after 25 min of heating and remained constant up to 40 min (Figure 8).

**Figure 5.** Effect of pH on the formation of RXN-NBD complex.**Figure 7.** Effect of temperature on the formation of RXN-NBD complex.**Figure 6.** Effect of concentration of NBD on the formation of RXN-NBD complex.**Figure 8.** Effect of time on the formation of RXN-NBD complex.

of PDAC, H_2SO_4 and effect of reaction time for the maximum color development. The results are incorporated in (Table 3) (Figure 9-11).

Method validation

The developed methods were validated by following ICH guidelines [50].

Beer's law and sensitivity: Calibration graph for the quantification of RXN was prepared under the optimum experimental conditions. Method I obeys Beer's law in the concentration range of 2-20 $\mu\text{g/mL}$ with the regression equation $A_{405} = 0.0348x + 0.0103$ (x =concentration of RXN in $\mu\text{g/mL}$). The Sandell's sensitivity and molar absorptivity of method A are $2.73 \times 10^{-3} \mu\text{g/cm}^2$ and $1.590 \times 10^5 \text{ L/mol/cm}$ of RXN, respectively.

Method II obeys Beer's law in the concentration range of 10-100 $\mu\text{g/mL}$ with the equation $A_{545} = 0.0061x + 0.0087$ (x =concentration of RXN in $\mu\text{g/mL}$). The Sandell's sensitivity and molar absorptivity of method B are $1.48 \times 10^{-2} \mu\text{g/cm}^2$ and $2.929 \times 10^4 \text{ L/mol/cm}$ of RXN, respectively.

The regression coefficient value of the standard curves for methods I and II were found to be 0.9993, showing good linearity of the

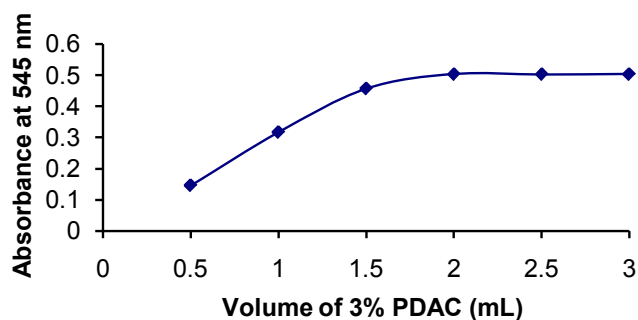
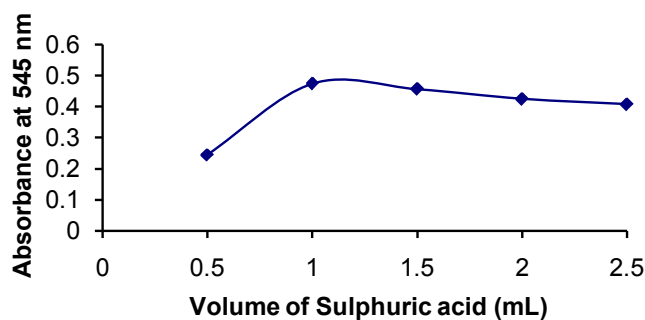
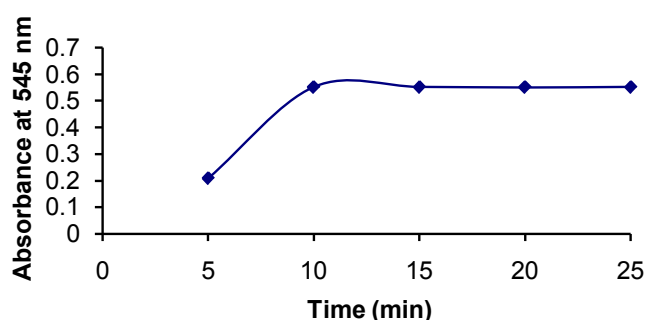
developed methods. The calculated limits of detection and quantitation values were 0.110 $\mu\text{g/mL}$ and 0.333 $\mu\text{g/mL}$, respectively, for method I, and their respective values for method II were 0.483 $\mu\text{g/mL}$ and 1.463 $\mu\text{g/mL}$.

Precision and accuracy: The precision and accuracy of the proposed methods I and II were determined using intra-day and inter-day analyses of three standard concentrations of RXN (method I – 2, 10 and 20 $\mu\text{g/mL}$; method II – 25, 60 and 125 $\mu\text{g/mL}$) in replicates ($n = 5$). The results are shown in Table 4. The percent relative standard deviation and percent recovery values show that the precision and accuracy, respectively, is satisfactory for both the proposed methods Table 4.

Recovery study: The accuracy of the proposed methods was further evaluated using recovery studies. Samples of tablet solution were spiked with pure RXN at three concentration levels (50,100 and 150 % of that in tablet). The total concentration of RXN was determined by the proposed methods. The results of recovery studies were given in Table 5. The percent recovery values indicated that the recovery was good. The co-formulated substances in the tablet did not interfere in the determination of RXN by the proposed methods.

Table 3. Optimization of conditions for the assay of RXN by Method II

Parameter	Investigation conditions	Optimized condition	Remarks
Volume of 3% PDAC (mL)	0.5-3.0	2.0	One mL of PDAC gave the highest absorbance value and there is no change in the absorbance with further raise in the volume (Figure 9).
Volume of H ₂ SO ₄ (mL)	0.5-2.5	1.0	The absorbance was increased with increasing volume of H ₂ SO ₄ and became constant at 1.0 mL; above this volume, the absorbance slightly decreased (Figure 10).
Effect of reaction time (min)	5 – 25	10	Maximum absorbance was obtained after 10 min and remained constant with increase in time (Figure 11).

**Figure 9.** Effect of concentration of PDAC on the formation of RXN-PDAC complex.**Figure 10.** Effect of concentration of sulphuric acid on the formation of RXN-PDAC complex.**Figure 11.** Effect of time on the formation of RXN-PDAC complex.

Selectivity: The selectivity of the proposed methods was established by observing any interference from the common tablet excipients. A placebo blank containing starch (20 mg), hydroxyl cellulose (25 mg), acacia (15 mg), lactose (20 mg), sodium citrate (30 mg), talc (20 mg), sodium alginate (25 mg) and magnesium stearate (25 mg) was prepared by mixing all the excipients into a homogeneous mixture. 100 mg of the placebo blank was precisely weighed and transferred to a volumetric flask (100 mL) and its solution was prepared as explained under section "Procedure for the analysis of RXN in tablet dosage forms". The

Table 4. Evaluation of precision and accuracy of the proposed methods (*Average of five determinations).

Method	Concentration of RXN (µg/mL)		Recovery (%)	RSD (%)
	Taken	Found*		
Intra-day analysis				
I	2	1.985	99.25	0.252
	10	9.962	99.62	0.371
	20	20.065	100.32	0.294
II	25	24.982	99.92	0.460
	60	60.115	100.19	0.606
	125	124.955	99.96	0.627
Inter-day analysis				
I	2	2.005	100.25	0.299
	10	10.055	100.55	0.487
	20	19.986	99.93	0.485
II	25	24.995	99.98	0.408
	60	59.945	99.91	0.692
	125	124.855	99.88	0.534

Table 5. Results of recovery study of the proposed methods (*Average of five determinations).

Method	Nominal amount (mg/tablet)	Amount of RXN added (mg)	Found*	RSD (%)	Recovery (%)
A	10	5	15.036	0.652	100.24
	10	10	19.952	0.621	99.76
	10	15	24.963	0.941	99.85
B	10	5	14.968	0.641	99.79
	10	10	20.102	0.900	100.51
	10	15	24.934	0.770	99.74

placebo blank solution was analyzed by following the general analytical methods I and II. The absorbance values of placebo blank solution and the reagent blank solutions of the proposed methods were almost equal. The results obtained from analysis of placebo blank shown that common excipients used in the tablet preparation did not obstruct the assay of RXN.

Robustness: The robustness of proposed methods was tested by changing the following experimental parameters:

Method I

- Buffer pH (7.8 ± 0.2)
- Volume of 0.1% NBD (1.0 ± 0.1 mL)
- Temperature (60 ± 2 °C)
- Reaction time (25 ± 2 min)
- Volume of HCl (0.2 ± 0.05 mL)

Method II

- Volume of 3 % PDAC (2.0 ± 0.2 mL)
- Volume of H₂SO₄ (1.0 ± 0.1 mL)

Table 6. Evaluation of robustness of the proposed methods (*Average of three determinations).

Method	Parameter	Concentration of RXN (µg/mL)		Recovery (%)	RSD (%)
		Taken	Found*		
I	Buffer pH (7.8 ± 0.2)	2	2.021	101.05	0.445
		20	19.962	99.81	0.606
	Volume of 0.1% NBD (1.0 ± 0.1 mL)	2	1.988	99.40	0.505
		20	20.014	100.07	0.569
	Temperature (60 ± 2 °C)	2	2.035	101.75	0.640
		20	19.949	99.75	0.631
	Reaction time (25 ± 2 min)	2	1.965	98.25	0.765
		20	20.046	100.23	0.688
II	Volume of 3 % PDAC (2.0 ± 0.2 mL)	2	1.978	98.90	0.964
		20	20.041	100.21	0.763
	Volume of H ₂ SO ₄ (1.0 ± 0.1 mL)	25	25.055	100.22	0.754
		125	124.965	99.97	0.613
	Reaction time (10 ± 2 min)	25	24.962	99.85	0.833
		125	124.955	99.96	0.674
		25	24.935	99.74	0.786
		125	124.929	99.94	0.530

Table 7. Evaluation of ruggedness of the proposed methods (*Average of three determinations).

Method	Parameter	Concentration of RXN (µg/mL)		Recovery (%)	RSD (%)
		Taken	Found*		
I	Analyst I	2	1.987	99.35	0.604
		20	20.054	100.27	0.573
	Analyst II	2	2.005	100.25	0.399
		20	19.944	99.72	0.476
	Instrument I	2	1.985	99.25	0.756
		20	19.826	99.13	0.570
	Instrument II	2	1.995	99.75	0.501
		20	20.070	100.35	0.827
II	Analyst I	25	25.015	100.06	0.628
		125	124.568	99.65	0.448
	Analyst II	25	24.755	99.02	0.686
		125	125.042	100.03	0.521
	Instrument I	25	24.96	99.84	0.781
		125	124.445	99.56	0.387
	Instrument II	25	24.995	99.98	0.816
		125	125.065	100.05	0.454

Table 8. Determination of RXN in tablets by the proposed methods (*Average of five determinations).

Method	Labeled claim (mg/tablet)	Found*	RSD (%)	Recovery (%)
I	10	9.965	0.722	99.650
II	10	10.024	0.738	100.240

➤ Reaction time (10 ± 2 min)

The robustness of the methods was evaluated at two different concentration levels (method I – 2 and 20 µg/mL; method II – 25 and 125 µg/mL). The results are shown in Table 6. The results showed no statistical differences suggesting that the proposed methods were robust.

Ruggedness: Ruggedness of the proposed methods was determined by the analysis of RXN standard solutions (method I – 2 and 20 µg/mL; method II – 25 and 125 µg/mL) by two different instruments and two different analysts using the same experimental conditions. The selected variables evaluated in the ruggedness testing are given in Table 7. No significant statistical difference was observed in the results, thus demonstrating that the proposed methods were rugged.

Application of the proposed methods to tablets: Both the methods were applied successfully to the analysis RXN in tablets (Xeralto tablets, labeled to contain 10 mg of RXN per tablet). Satisfactory results were obtained (Table 8). The results are in good agreement with label claim. The proposed methods, therefore, are suitable for the determination of RXN in tablets with adequate accuracy and precision.

Conclusion

The present study reported the successful evaluation of 4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (method I) and *p*-Dimethylaminocinnamaldehyde (method II) as chromogenic reagents in the development of two spectrophotometric methods for the precise and accurate determination of RXN in bulk and in its tablet dosage forms. The developed methods are fully validated as per the ICH guidelines. The developed methods do not need expensive sophisticated apparatus. The proposed methods are sensitive, selective, robust, rugged, precise and accurate. The colored complexes produced in methods I and II remains stable for at least 60 min. This gives the high throughput property to the proposed methods. Therefore, the

methods A and B are valuable for its routine application in quality control laboratories for analysis of RXN.

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Conflict of interest

The authors have no conflict of interest in this study.

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