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Determination of Rivaroxaban in pure, pharmaceutical formulations and human plasma samples by RP-HPLC

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Abstract

A simple, sensitive and rugged reverse-phase high performance liquid chromatographic method has been developed and validated for the determination of rivaroxaban in pure, pharmaceutical formulations and in spiked human plasma sample. The separation of rivaroxaban and internal standard was achieved on XDB C18 (150 x 4.6) mm column. Mobile phase employed for the study is the mixture of water and acetonitrile in gradient programme. A flow rate of 1 ml/min was found optimum for the study. Linearity of the proposed method was found to be in the range of 0.05 µg/ml to 20 µg/ml with r^2=0.9999. The limit of detection and limit of quantification of the proposed method are found to be 0.015 µg/ml and 0.046 µg/ml, respectively. Intra-day and inter-day assay relative standard deviations were determined and found to be less than 2.0 %. The method has been applied successfully for the determination of rivaroxaban in its pharmaceutical formulations and in spiked human plasma samples.

Keywords: Rivaroxaban, RP-HPLC, Validation, Dosage forms, Human plasma.

1. Introduction

Rivaroxaban (RVX) is an oral anticoagulant, which prevents clotting of blood. The IPUAC name for RVX is (*S*)-5-chloro-*N*-{[2-oxo-3-[4-(3-oxomorpholin-4-yl) phenyl]oxazolidin-5-yl]methyl} thiophene-2-carboxamide. The chemical formula of RVX is as shown in Fig. 1. RVX is a reversible, direct factor Xa inhibitor that is rapidly absorbed with maximum concentrations in healthy and elderly subjects.[1, 2] RVX is found to have anti-thrombotic effects.[3] Thrombin multiplication occurs in Factor Xa at the rate of 1000th fold.[2, 4] Hence, factor Xa plays an important role in treating the thromboembolic disorders. The inhibition of factor Xa by RVX is due to its binding with S1 and S4 pockets of the enzyme serine endopeptidase with high selectivity. [5] Inhibition of factor Xa interrupts the intrinsic and extrinsic pathway of the blood clotting process, inhibiting both thrombin formation and thrombi development.

In the literature only a few methods for the determination of RVX has been reported. They include Spectrophotometry, [6] HPLC, [7-11] HPTLC, [12] and LC-MS. [13] None of the reported methods doesn't study the determination of RVX in human plasma, whereas the proposed method describes the HPLC method for the determination of RVX in pure, pharmaceutical formulations and spiked human plasma samples.

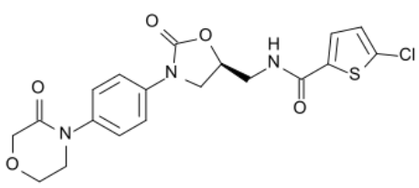

Figure 1: Rivaroxaban

2. Experimental

2.1 Reagents and Chemicals

HPLC grade acetonitrile and methanol were purchased from Sigma, purified de-ionized water was prepared in the laboratory using Barnstead Nano-pure Diamond water purification system (Thermo, Waltham, MA). RVX and vemurafenib were kindly provided by Apoptex Pharmachem India Pvt Ltd., Bangalore, as gift samples. Commercial dosage forms of RVX were purchased from the local market.

2.2 Instrumentation

The Waters 2690 HPLC system consisted of gradient pump with degassing facility and gradient mixer, photodiode array detector, an auto sampler and an Empower workstation was employed for the study. Chromatographic separation was achieved on Agilent, Zorbax Eclipse XDB C_{18} (150 x 4.6) mm, connected with a guard column associated with the same bonded phase. The mobile phase consisted of water (A-pump) and acetonitrile (B-pump) in the gradient program as follows: Time (min) / B (%): 0/35, 7.3/35, 8.0/65, 12/65, 12.1/35, 14/35. 1 ml/min of flow rate was maintained throughout the chromatographic run, i.e., 14 min. Sample temperature and column temperature were maintained at 15 ^{0}C and injection volume was 20 µl.

2.3 Preparation of Standard Solution

RVX stock solution containing 1 mg/ml was prepared in methanol:acetonitrile in the ratio of 1:1 and a stock solution of vemurafenib (Internal Standard, IS) containing 1 mg/ml was prepared in the mixture of water and acetonitrile in the ratio of 2:8. The solutions were diluted as when required. Standard working solutions were prepared individually in the diluent, methanol:acetonitrile (1:1). Working solution of each of 250 µg/ml of RVX and IS were prepared separately in the diluent.

2.4 Preparation of Pharmaceutical Formulation

Twenty tablets of RVX were finely powdered and an amount equivalent to the 25 mg of the drug was weighed accurately and transferred into a 100 ml beaker. The powdered drug was completely disintegrated in the diluent, using a mechanical stirrer. The solution was filtered through 0.45 µ filter paper and was diluted upto the mark of 50 ml with the diluent.

2.5 Preparation of Spiked Human Plasma Sample

Saline and sodium citrate solutions were taken in dry and evacuated tubes. Human blood samples from different healthy volunteers were collected in dry and evacuated tubes, after overnight fast, before breakfast. Within 1h of plasma collection, the samples were centrifuges at 1600 rpm for 12 min. The plasma samples were spiked with RVX and IS and were extracted with ether. The ethereal layer was evaporated to dryness on a water bath at 40 ^{0}C under the low stream of nitrogen gas. The residue was dissolved in the diluents, stored at -20 ^{0}C until further analysis.

2.6 Validation of the Method

The validation of the proposed HPLC method was carried out according to the Food and Drug Administration (FDA) guidelines. [14] Linearity of the proposed method was determined by replicate analysis of 6 complete standard curves on 6 different days. A linear regression was

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used to plot the peak area ratio (y) of RVX to IS *versus* RVX concentration. Intra- and inter-day precision and accuracy expressed as bias were evaluated at three levels of concentrations. Six replicates of each level were assayed in one run for the intra-day experiment. Three replicates of each level were assayed within six different days for the inter-day experiment. Average recovery of RVX was determined by comparing the peak area at different concentration levels (6 replicates for each level) with those obtained by direct injection of the same amount of drug.

3. Results and Discussion

3.1 Chromatographic Method Development

Various compounds *viz*., amoxicillin, cefedroxil, valganocyclovir, andrographolide, vemurafenib were tested for the purpose of internal standard, but a good chromatographic characteristics like ideal retention time, resolution, peak shape, etc were observed with vemurafenib and hence it was selected as an internal standard. The mobile phase was chosen after various trials with methanol, water, istopropyl alcohol, acetonitrile, triethylamine and various buffers with different pH in various proportions. Because of high transparency in UV region, acetonitrile was selected with water as the mobile phase. For the selected mobile phase in the above mentioned gradient program, both RVX and internal standard gave good chromatographic separation and behavior. The effect of flow rate was examined by varying the flow rate of the mobile phase from 0.75 ml/min to 1.50 ml/min and observed that a flow rate of 1.0 ml/min gave optimal signal to noise ratio with a reasonably good separation time and hence a flow rate of 1.0 ml/min was chosen for the study. The maximum absorption of RVX and IS together were found to be at 250 nm and hence this wavelength was selected for the experiment. Using C_{18} column, the retention times of RVX and IS were found to be 5.08 min and 11.95 min respectively. A typical chromatogram obtained is shown in Fig. 2.

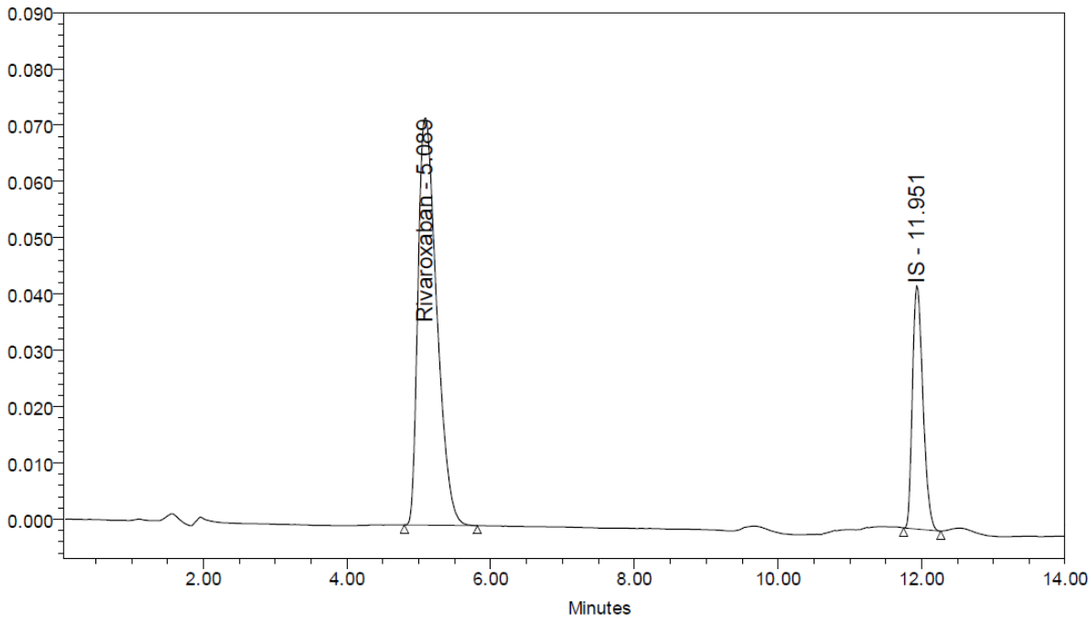

Figure 2: A typical chromatogram showing the separation of RVX and IS

3.2 Suitability of the Proposed Method

The system suitability of the proposed method is determined. The suitability parameters like resolution, peak asymmetry, capacity factor, theoretical plates were calculated and are tabulated in Table 1. For a system to be suitable for the determination of an analyte, the resolution should be more than 1.5, peak asymmetry should be between 0.8 and 1.2, and selectivity should be more than 1.0. The system suitability values of the proposed method revealed that, the method is ideal for the determination of RVX in pure, bulk and spiked human plasma samples.

Table 1: System suitability parameters

Parameter	RVX	IS
Number of theoretical plates	1589	19778
Retention time (t_R) in min	5.08	11.95
Resolution		27.48
Capacity factor (k')	6.47	16.57
Selectivity factor (α)		2.56
Peak asymmetry (A_S)	1.08	1.03
Height equivalent to theoretical plate (H) in mm	0.09	0.01

3.3 Selectivity

The specificity of the proposed method was examined by the injection of different drugs such as andrographolide, valganocyclovir, erlotinib, amoxicillin, sorafenib cefedroxil, and found they did not interfere in the determination of RVX, which was evident from their retention times which are different from that of RVX.

3.4 Linearity

Linear relationship between the peak area ratio of RVX and IS corresponding to concentration of RVX was observed. Linearity of the method was determined by analyzing different solutions containing 0.05 µg/ml to 20 µg/ml of RVX and fixed concentration of internal standard (5 µg/ml), under the mentioned chromatographic conditions. Six independent determinations were carried out at each concentration level. Table 2 gives the regression line, correlation coefficient, slope, intercept and % RSD. An excellent linearity was observed in the range of 0.05 - 20 µg/ml, as evident by its r^2 value, 0.9999.

Table 2: Linearity, LOD and LOQ

Parameter	Value
Linearity range (µg/ml)	0.05 - 20
Regression equation (Y)[a]	
Slope (b)	0.066
Intercept (c)	0.004
Correlation coefficient (r^2)	0.9999
LOD (µg/ml)	0.015
LOQ (µg/ml)	0.046
% RSD	1.64

[a]Y = Bx + C, where, x is the concentration of RVX in µg/ml.

3.5 Limit of Detection and Limit Quantification

The limit of detection (LOD) was established at a signal-to-noise ratio (S/N) of 3 and the limit of quantification (LOQ) was calculated at S/N value of 9. The LOD and LOQ values were found to be 0.015 µg/ml and 0.046 µg/ml, respectively. The values are shown in Table 2.

3.6 Precision and Accuracy

The precision of the proposed method was examined by carrying out the analysis on within-day and between-day variations of determinations. This was done by injecting the RVX at its LOQ level (n=6). The accuracy of the proposed method was determined as follows: a standard working solution containing 10 µg/ml of RVX and 5 µg/ml of internal standard was injected and the chromatograms were recorded. This was repeated for 6 times (n=6). From the respective area counts, the concentrations of RVX were calculated using linearity curve. The accuracy, defined in terms of percentage deviation of the obtained concentration from the actual concentration was found to be satisfactory. The precision and accuracy data obtained are tabulated in Table 3.

Table 3: Precision and accuracy data for the determination of RVX

Actual concentration, µg mL^{-1}	Intra-day concentration measured (n=6)	% RSD	% Bias	Inter-day concentration measured (n=3)	% RSD	% Bias
5	5.03	1.423	0.60	5.06	1.468	1.20
10	9. 92	1.658	- 0.80	9.96	0.859	- 0.40
20	19.88	0.925	- 0.60	19.93	1.067	- 0.35

3.7 Recovery Studies

To study the recovery of the proposed method, 80%, 100% and 120% of RVX was added to a pre-analyzed sample. The recovery studies were carried out three times over the specified concentration range and the percentage recovery was found to be in the range of 98.85% to 100.82%.

3.8 Ruggedness of the Method

The ruggedness of the proposed HPLC method was examined by carrying out the analysis of the working standard solution by employing the same chromatographic conditions and system on different days good consistency in retention times and very negligible differences in areas were noticed after 48h time period. The % RSD values were found to be less than 2.0 for both retention times and areas. The good detector responses obtained on different days showed that the proposed method is rugged. The results are showed in Table 4.

Table 4: Ruggedness of the method

Parameter	RVX		IS	
	1st day	2nd day	1st day	2nd day
Area[a]	1328146	1331328	438037	4391029
SD	14875.24	20103.05	7578.04	72012.88
% RSD	1.12	1.51	1.73	1.64
Retention time[a]	5.082	5.076	11.953	11.947
SD	0.057	0.064	0.090	0.109
% RSD	1.06	1.26	0.75	0.91

[a] Average values of six determinations.

4. Applications

4.1 Analysis of Pharmaceutical Formulations

The proposed analytical method was applied successfully for the analysis of RVX in its pharmaceutical formulations. The low values of percentage relative standard deviation indicated that the method is highly precise for the analysis of RVX in its formulations. The results obtained are tabulated in Table 5.

Table 5: Analysis of RVX in its dosage forms

Tablet	Strength, mg	Found[a], mg	% RSD	% Recovery
Xraelto	10	10.08 ± 0.71	0.55	100.80
	15	14.89 ± 0.94	1.28	99.27
	20	19.95 ± 0.82	1.66	99.75

[a] Mean value of six determinations.

4.2 Analysis of Plasma Samples

The proposed method was applied successfully for the analysis of RVX in spiked human plasma samples. The obtained results for the accuracy and precision at three different levels in spiked plasma are tabulated in Table 6. Low values of % RSD and % Bias revealed that the proposed method is highly precise and accurate.

5. Conclusion

A reverse phase HPLC method for the determination of RVX in pure, dosage forms and spiked human plasma has been developed and validated. The proposed method showed good accuracy and precision and acceptable linearity. By employing the proposed HPLC method, one can detect the RVX as low as 0.015 µg/ml. The proposed method is rapid in determining the RVX in plasma sample, since plasma sample preparation before chromatographic analysis is quite simple. The proposed HPLC method could be employed to analyze a large number of plasma samples each day in clinical laboratories and pharmaceutical dosage forms in routine quality control laboratories. Hence the proposed method could be adopted for the assay of RVX in plasma samples and pharmaceutical formulations.

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