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Stability indicating RP-HPLC method development and validation for the estimation of Betrixaban (BET) in bulk and its pharmaceutical formulations

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ABSTRACT

A simple, rapid, sensitive and most economical RP-HPLC method has been developed and validated for the estimation of Betrixaban in bulk and its tablet dosage form. Separation was performed on a Water's C_{18} (250 cm×4.6 mm×5µm) column at ambient temperature. The mobile phase consisted of Water and ACN in the ratio of 88:12v/v at a flow rate of 1mL/min. The analyte was determined using a photodiode array detector at a detection wavelength of 272nm. The retention time of Betrixaban was found to be 3.284min. The validation of the proposed method was carried out for specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness. The linear dynamic range was from 70 – 210 µg/mL. The percentage recovery obtained for Betrixaban was100.10%. Limit of detection and quantification were 4.96 and 16.52 µg/mL. The developed method can be used for routine quality control analysis of titled drug in tablet formulation.

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INTRODUCTION

Betrixaban is a non-vitamin K oral anticoagulant drug, whose action is driven by the competitive and reversible coagulation factor Xa. It was selected among all lead compounds due to its low Herg channel affinity while sustaining its factor Xa inhibition capacity. Betrixaban is prescribed as a Venous Thromboembolism (VTE) prophylactic for adult patients with moderate to severely restricted motility or with other risks for venous thromboembolism. Venous thromboembolism can be manifested as deep vein thrombosis or pulmonary embolism and it is a leading cause of preventable death in hospitalized patients. Betrixaban, (Figure1) is chemically N-(5-chloropyridine-2-yl)-2-[[4-(N,N- dimethylcarbamimidoyl)benzoyl] amino]-5-methoxybenzamide with molecular formula of $C_{23}H_{22}ClN_5O_3$ and molecular weight of 451.911 gm/mol. pKa of 11.63, marketed with the trade name Bevyxxa-80mg manufactured by Taj Pharmaceuticals India, and is soluble in water and methanol, primarily excreted unchanged.

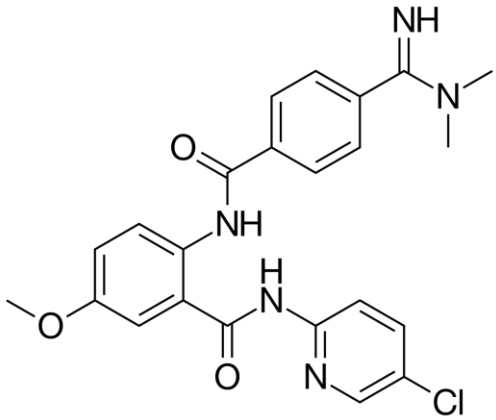

Figure 1: Chemical structure of Betrixaban

A thorough literature survey has been done and found only one RP-HPLC method (SK

Masthanamma *et al*., 2018) for the determination of Betrixaban in the single dosage form.

EXPERIMENTAL INVESTIGATION

Instrumentation

The HPLC system consisted of a LC Waters (Waters, Milford, MA, USA) using a Water's C_{18} 250cm×4.6mm× 5µm column, a quaternary gradient system (600 Controller), in line degasser (Waters, model AF24). The system was equipped with a photodiode array detector (Water**s**, 2998 model) and autosampler (Waters, model 717 plus). Data were processed using Empower 2 software (Waters, Milford, MA, USA). Water:ACN in the ratio of 88:12v/v was used as a mobile phase which was pumped at a flow rate of 1.0 mL min−1. The detection wavelength was 272 nm.

Chemicals and Reagents

The reference standard of Betrixaban was kindly supplied by Taj Pharmaceuticals, India with purity of 99.76%. Tablet formulation containing Bevyxxa-80mg was procured from Taj Pharmaceuticals India. Acetonitrile (HPLC grade) was purchased from Spectrochem (Mumbai, India). All other reagents and chemicals used in this study were of analytical grade. Water was purified using a Millipore system (Millipore Corp., Bangalore, India).

Preparation of Stock and Standard Solutions

The standard stock solution containing 1 mg /mL (1000 µg/mL) of Betrixaban was prepared by dissolving 100 mg of the reference standard in 100mL mobile phase (Water and ACN in the ratio of 82:12 v/v) and diluting with the same diluent. 1.4 mL aliquot of Betrixaban was transferred to 10 mL calibrated volumetric flask and the volume was made up to the mark with the same solvent mixture to prepare a standard solution having a concentration of 140µg/mL of Betrixaban. Calibration standards containing 70.0 to 210.0µg/mL of Betrixaban was prepared by diluting the standard stock solution to the appropriate volume with the same diluent.

Preparation of Test Solution

Twenty tablets were weighed and finely powdered in a mortar. Tablet powder equivalent to 10 mg of Betrixaban was accurately weighed and transferred to a 10 mL calibrated volumetric flask dissolved in mobile phase mixture, and the solution sonicated for 10 min. Volume was made up to the mark with the same solvent to get sample solution of 1000 µg/mL. The solution was filtered through 0.45 µm membrane filter, 1.40mL of the solution was diluted to 10mL with the diluent. This solution contains 140µg/mL. 10µL of the resulting solution was injected into the chromatograph. Peak area and Rt were determined from chromatogram and the amount of Betrixaban was calculated.

Stability studies

All forced degradation studies (Stability studies) were performed at an initial drug concentration. Acid hydrolysis was performed in 0.1N HCl at 105°C for 3H. Alkali hydrolysis was carried out in 0.1 N NaOH at 90°C for 3 H. Oxidative studies were performed at 90^0 C in 3% hydrogen peroxide for 4 H. UV degradation studies were carried out at 257nm for 25H. For thermal degradation, drug powder was heated to 80°C for 24H. Samples were withdrawn at appropriate times and subjected to HPLC analysis after suitable dilution.

Validation of the Method

The developed method was validated as per the International Conference on Harmonization (ICH) guidelines with respect to Precision, Accuracy, Linearity, robustness, LOD & LOQ.

Precision

Six injections, of optimized concentrations of the analyte (140µg/mL), was given on the same day and the values of relative standard deviation (%RSD) were calculated to determine intra-day precision. These studies were also repeated on different days to determine inter-day precision.

Accuracy

Accuracy was evaluated by fortifying a mixture of the dosage form with three known concentrations of the drug. The percent recovery of the drug from the dosage form was determined.

Linearity

A stock solution of the drug was prepared at the strength of 1000 µg/mL. It was further diluted to prepare solutions containing 70.0 to 210.0 µg/mL of Betrixaban. The solutions were injected in triplicate into the HPLC column, keeping the injection volume constant (10µL).

Specificity and selectivity

The specificity of the method was established through the study of resolution factors of the drug peak from the nearest resulting peak, and also among all other excipients.

Robustness

Robustness of the study was evaluated by changing the parameters of the method like flow rate (±0.2 ml) and column temperature (± 5°C).

Detection and quantitation limits

Standard solutions were prepared by sequential dilutions and injected on the chromatograph, at

Table 1: Optimized chromatographic conditions for determination of Betrixaban

S. No	Parameter	Description/Value
1	`	Water's C_{18} (250×4.6×5)
3	Flow rate	1 mL/min
4	Detection Wavelength	272 nm
5	Detector	Photodiode array
6	Injection	Autosampler -Waters, model 717 plus
8	Injection volume	10 µL
9	Column Temperature	Ambient
10	Runtime	5 mins
11	Diluent	Mobile Phase

Table 2: System suitability data of Betrixaban

S. No	Parameter*	Betrixaban
1	Theoretical Plate Count	6891
2	Average Peak Area	7919001
3	Peak Height	1152601
4	RT	3.284
5	Tailing	1.0
6	Resolution	-
7	S/N	1045

* Average of 6 replicates

Table 3: Accuracy Results of Betrixaban

Accuracy Level	Wt. of Sample	Peak Area	Amount Added	Amount Found	% Recovery	Mean % Recovery
50%	15.710	3631749	63.73	64.21	100.75	100.85
	15.710	3611606	63.73	63.85	100.19	
	15.710	3673683	63.73	64.95	101.91	
	15.710	3630428	63.73	64.18	100.71	
	15.710	3613458	63.73	63.88	100.24	
	15.710	3652716	63.73	64.58	101.33	
100%	31.42	7194479	127.46	127.19	99.79	99.94
	31.42	7161061	127.46	126.60	99.32	
	31.42	7261150	127.46	128.37	100.71	
150%	47.130	10739927	191.19	189.87	99.31	99.52
	47.130	10708563	191.19	189.32	99.02	
	47.130	10785958	191.19	190.68	99.73	
	47.130	10875024	191.19	192.26	100.56	
	47.130	10863028	191.19	192.05	100.45	
	47.130	10604679	191.19	187.48	98.06	
Average % Recovery						100.10

Table 4: Precision results of Betrixaban

S. No	Peak Area	
	Intraday precision	Interday precision
1	7122699	7149579
2	7242606	7289022
3	7218974	7289022
4	7176367	7216189
5	7265881	7216189
6	7232006	7154002
Average	7209755.50	7219000.50
SD	51967.14	61426.35
% RSD	0.72	0.85

decreasing concentrations. The limit of detection was defined as the concentration for which a signal to noise ratio of 3 was obtained and for quantitation limit, a signal to noise ratio of 10 was considered.

RESULTS AND DISCUSSION

In order to achieve elution of the drug, different chromatographic conditions were attempted. Stationary phases like C_8, C_{18} and cyano were tested. Betrixaban eluted in all the stationary phases, while it was retained with C_8 and cyano columns using different mobile phase compositions of water and acetonitrile (65:35, 70:30, 85:15, 90:10 and 95:5 (v/v)). The drug was eluted with C_{18} column. To reduce runtime, the mobile phase composition was selected as water: acetonitrile, in the ratio of 88:12 (v/v). To reduce the analysis time the gradient system was also employed but the peak area reproducibility for both the analytes was found to be very poor. Isocratic elution with water:ACN 88:12 v/v was finally optimized. Table 1 shows the Optimized Chromatographic conditions for the determination of Betrixaban.

Under these optimized conditions, the analyte peak was well resolved and free from tailing. The tailing factor was <1.5 for the peak, at a flow rate of 1.0 mL/min. The column temperature was maintained ambient. The chromatogram was recorded at 272 nm with a PDA detector. Representative chromatograms of blank, standard and tablet extract were recorded and shown in Figure 2-4.

Method Validation

The developed method was validated as per ICH guidelines Q2 (R1). The validation parameters studied were system suitability, specificity, linearity, accuracy, precision (Intraday and Interday), limit of detection, limit of quantitation, robustness and forced degradation studies.

System Suitability

Prior to the method validation study, system suitability tests were performed by measurement of general characteristics such as peak symmetry, the number of theoretical plates, retention time, tailing factor etc. the results obtained were satisfactory and in accordance with guidelines. The system suitability was evaluated by six replicate analyses of analytes at 140 µg/mL of Betrixaban. Average theoretical plate count was found to be 6891. The retention time of Betrixaban was found to be 3.284 min. Results were shown in Table 2.

Specificity

Specificity of an analytical method is its capability to measure the analyte precisely and particularly in the presence of parts that may be likely to be present in the sample matrix. Chromatograms of standard and sample (Figure 3-4) show the no interference due to blank and Excipients which prove that the method was specific.

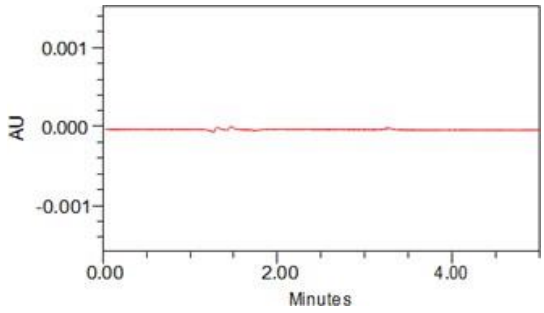

Figure 2: Blank chromatogram of Betrixaban

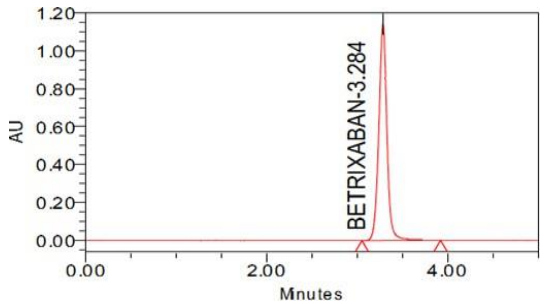

Figure 3: Standard Chromatogram of Betrixaban

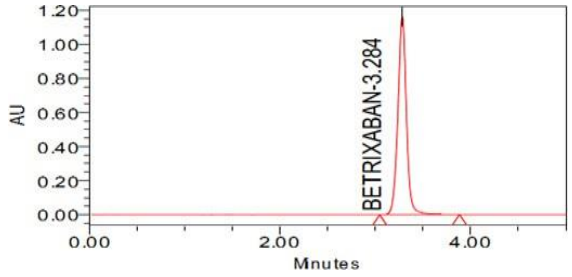

Figure 4: Sample Chromatogram of Betrixaban

Accuracy

The accuracy of the method was determined by the % recovery method at three concentration levels (50%, 100% and 150%) of the test solution. Six replicates were analyzed for 50 % and 150 %, 3 replicates were tested for 100%. The mean recovery of Betrixaban was found to be in between 99-100 %. Table 3 shows the results of accuracy and chromatograms of accuracy study were shown in Figure 5.

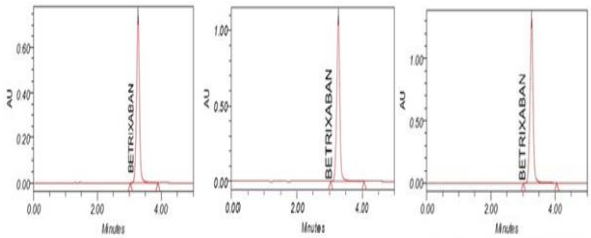

Figure 5: Accuracy chromatograms of Betrixaban

Precision

Inter and Intra-day precision of the method was determined by performing precision three times on the same day and followed by three consequent days. % RSD was calculated and found to be within the specified limits (<2%), which proves that the developed method was precise. Table 4 shows the precision results and chromatograms were depicted in Figure 6.

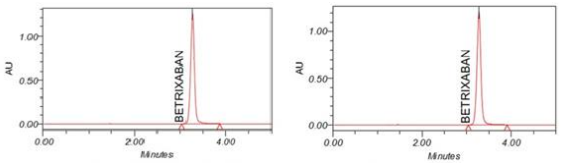

Figure 6: Intra and Inter-day precision chromatograms of Betrixaban

Linearity

Linearity was constructed with five concentrations at the level of 50-150% (70.00, 105.00, 140.00, 175.00, 210.00 µg/ml Betrixaban). The peak area of the analyte was found to be linear in the studied concentration range, the correlation coefficient was found to be 0.9999 for Betrixaban. The linearity results and curve was shown in Table 5 and Figure 7 respectively, and the Chromatograms of linearity was shown in Figure 8.

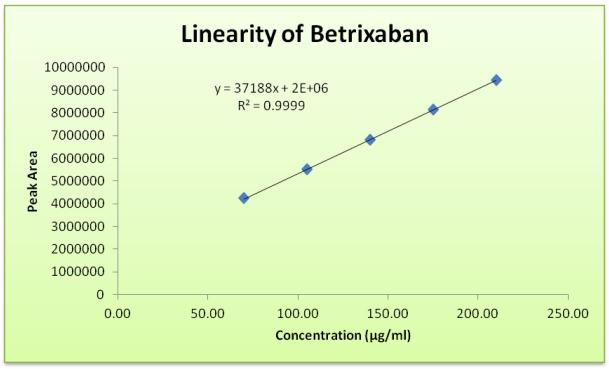

Figure 7: Linearity Plot of Betrixaban

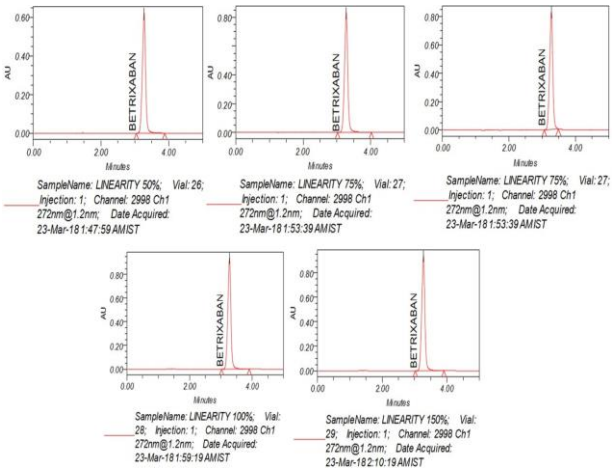

Figure 8: Linearity Chromatograms of Betrixaban

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined by using the standard deviation of response and slope of calibration curves. The LOD and LOQ for Betrixaban of the proposed method were found to be 4.96µg/mL and 16.52µg/mL respectively. Figure 9 shows the chromatograms of LOD and LOQ.

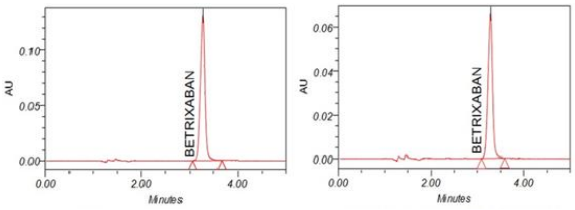

Figure 9: LOD and LOQ Chromatograms of Betrixaban

Robustness

The robustness of the analytical method was evaluated by assaying the test solutions after slight but deliberate changes in the conditions like flow rate (± 0.1 ml/min) and the column temperature (± 2°C). System suitability data was found to be satisfactory during variations of the analytical conditions. System suitability results were also remained unaffected by slight changes in the analytical conditions. Table 6 shows the results and chromatograms were shown in Figure 10.

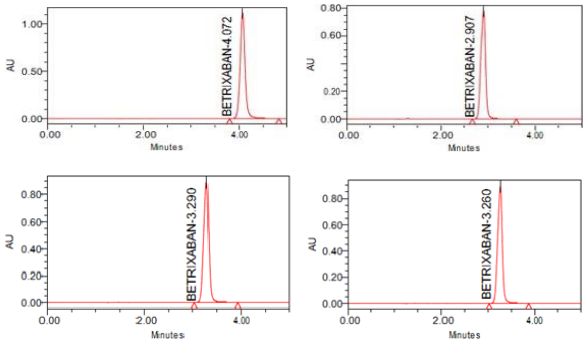

Figure 10: Robustness chromatograms of BET

Assay

The proposed method was applied for the tablets of Betrixaban, the mean % assay was found to be 99.73 % for Betrixaban. Results were given in Table 7.

Forced Degradation Studies (Stress testing):

Stress studies were performed to evaluate the stability indicating the ability of the developed RP-HPLC method by exposing the sample solution to different the stress conditions viz., acid, base, peroxide, UV and heat. Assay studies were carried out for stress samples at 150µg/mL against a reference standard. The proposed RP-HPLC method can able to detect the analyte even in the presence of

Table 5: Linearity data of Betrixaban

S. No	Linearity Level	Betrixaban	
		Concentration (µg/ml)	Peak Area
1	50	70.00	4242136
2	75	105.00	5496681
3	100	140.00	6794914
4	125	175.00	8123328
5	150	210.00	9436630
Reg. Equation		y = 37188x + 2E+06	
Slope		37188	
Y-Intercept		2E+06	
R^2		0.9999	

Table 6: Robustness Results of Betrixaban

S. No	Parameter	Condition	Betrixaban		
			RT	Peak Area	% Assay
1		0.8 ml/min	4.072	7157652	99.01
2	Flow	1 ml/min	3.284	7216189	99.81
3		1.2 ml/min	2.907	7308459	101.09
4		25 °C	3.290	7125339	98.56
5	Temp	30 °C	3.284	7216189	99.81
6		35 °C	3.260	7342750	101.57
	Average		3.35	7227763.00	99.97

Table 7: Assay Results of Betrixaban

S. No	Sample Weight (mg)	Peak Area	% Assay
1	31.42	7122699	98.52
2	31.42	7242606	100.18
3	31.42	7218974	99.85
4	31.42	7176367	99.26
5	31.42	7265881	100.50
6	31.42	7232006	100.03
Average		7209755.50	99.73
SD		51967.14	0.72
% RSD		0.72	0.72

Table 8: Forced degradation studies of Betrixaban

S. No	Condition	Betrixaban		
		Peak Area	% Assay	% Degradation
1	Acid	6549579	90.59	9.41
2	Base	6489022	89.76	10.24
3	H2O2	6389022	88.37	11.63
4	UV	6516189	90.13	9.87
5	Heat	6616189	91.52	8.48

degraded products and thus confirms the stability indicating the power of the developed method.

For acid degradation condition, drug solution was treated with 2.5 ml of 0.1 N HCl and heated for 105°C for 3 H, cooled and added 2.5mLof 0.1 N NaOH to neutralize any excess acid present in the sample. 10 µL of the sample solution was injected into HPLC. For base degradation, drug solution was treated with 2 mL of 0.1 N NaOH and heated for 90°C for 3 H, cooled and added 2mL of 0.1 N HCl to neutralize any excess base present in the sample. 10 µL of the sample solution was injected into HPLC. For peroxide degradation, drug solution was treated with 2.5 mL of 3 % H_2O_2 and heated for 90°C for 4 H, cooled and 10 µL of the sample solution was injected into HPLC. For UV degradation, drug sample was exposed to UV light in a UV chamber at 257 nm for 25 H. 10 µL of the sample solution was injected into HPLC. For heat degradation, drug solution was refluxed at 80°C for 24 H. 10 µL of the sample solution was injected into HPLC. Table 8 shows the results and chromatograms were shown in Figure 11.

CONCLUSION

Simple and efficient stability indicating RP-HPLC method has been developed and validated for the isocratic separation and assay determination of

Betrixaban in bulk and single dosage form. The proposed method, suitable for routine quality control, has been successfully applied to the determination of an analyte in commercial brands of tablets. This method is specified as the analyte peak is well resolved from the Excipients peaks with a total run time of 5 min.

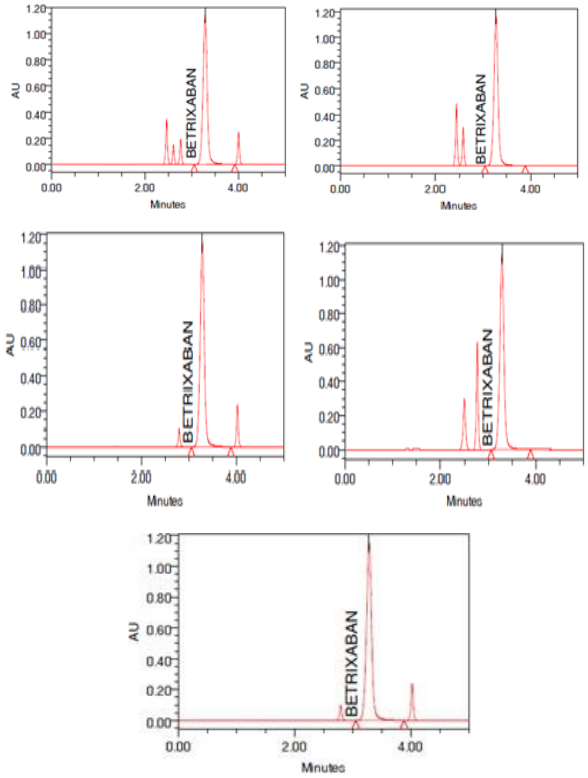

Figure 11: Forced Degradation studies of Betrixaban

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