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Development and validation of stability indicating method for estimation of Betrixaban in bulk and pharmaceutical formulation

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Abstract: **- Stability indicating reverse phase-high performance liquid chromatographic method is developed for the estimation of Betrixaban, an oral anticoagulant in formulation. HPLC separation was performed for the drug on the Hi-Q Sil C-18 column, 250 mm × 4.6 mm, 5.0 µ. A very simple mobile phase was used containing methanol: water (80: 20) v/v with a flow rate of 0.7 mL/min and analyte was examined at 272 nm by means of photodiode array (PDA) detector. Retention time of Betrixaban was found to be 2.91 min. For linearity study, the concentration range selected was 10-90 µg/mL and the calibration graph displayed a linear curve over the concentration vs area plot. The mean value of the correlation coefficient was 0.9998, slope was 8581.9 and intercept was 1989.1. The limit of detection (LOD) was 0.5980 µg/mL and limit of quantitation (LOQ) was 1.7381 µg/mL. RP-HPLC method was successfully applied to capsule formulation, no interference of drug and excipients was observed. The method was successfully validated as per ICH guideline in terms of precision, recovery and robustness. In stability studies, Betrixaban was exposed to various stressors; viz: acid, alkali, neutral, oxidation, photo degradation and dry heat. Betrixaban has shown more than 5 % degradation in alkaline, acidic, neutral and photo degradation studies; whereas it is showing less than 5 % degradation in oxidative and dry heat condition. The developed method being very simple and accurate can be applied for routine analysis of Betrixaban in pure and capsule formulation.**

Keywords: Method development, RP-HPLC, Betrixaban, ICH, Validation

1. INTRODUCTION

The Q1A (R2) guideline for stability testing of drug issued by the International Council for Harmonization (ICH)[1] recommends that stress studies must be performed on a drug substance and product for establishing inherent stability characteristics, which will lead to identify the degradation products. This will support the suitability of the proposed method and should be completely validated.

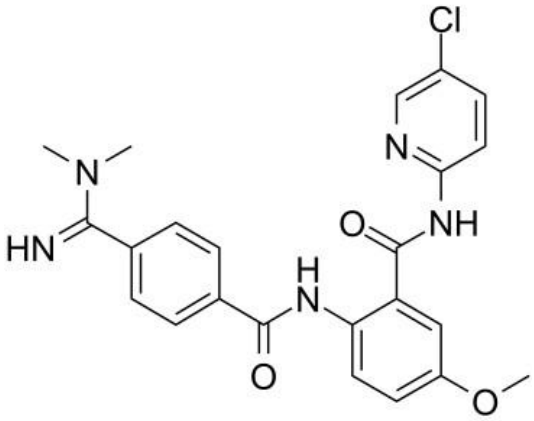

Fig. 1: Structure of Betrixaban

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Betrixaban, (N)-(5chloropyridin-2-yl)-2-((4-(-N),-(N)-dimethylcarbamimidoyl) benzoyl)amino)-5-methoxybenzamide (Fig. 1), is an oral anticoagulant drug (class III).[2] Betrixaban is an oral, reversible inhibitor of the coagulation factor Xa (FXa) having selective and direct action. Anticoagulant and antithrombotic effects are exerted by inhibition of FXa which leads to decrease in the conversion of prothrombin to active thrombin. This diminishes thrombin-mediated activation of the coagulation process leading to fibrin formation and activation of platelets.[3]

Literature review reveals that there are only two methods are reported for Betrixaban by HPLC which are stability indicating[4], [5], simultaneous estimation by liquid chromatography/tandem mass spectrometry (LC–MS/MS) using multiple reaction monitoring (MRM) is reported[6], hence there is a need for developing a stability indicating method for estimation of Betrixaban.

The present work aims in developing a specific, accurate and repeatable, stability-indicating HPLC method for determining Betrixaban in presence of related impurities and degradation products and to evaluate the purity of bulk drug and to establish stability of its bulk dosage forms. The proposed method was validated as per ICH guidelines[7]-[9] and its updated international convention[10].

EXPERIMENTAL

Materials

Hetero Pharma, Pvt. Ltd, India, supplied Betrixaban as a gift sample of purity 99.37 % (w/w) on dry weight basis. Capsule formulation of 80 mg was prepared in-house to perform the Assay**.** Reagents and chemicals used for analysis were of HPLC and analytical grade.

Instrumentation

Chromatographic separation was achieved on Jasco HPLC system (model PU 2089) with a PDA detector which was operated at a wavelength of 272 nm and rheodyne injector used was of 20 µL loop volume. For collecting the data and for processing, ChromNav software was used. Hi-Q Sil C-18 column of dimension 250 mm × 4.6 mm, 5 µm was used.

Preparation of mobile phase:-

Methanol and water were mixed in the ration of 80: 20. The degassing of mobile phase was done using an ultrasonic sonicator for 15 minutes to remove the dissolved gases present in the mobile phase and 0.45 µm whatmann's filter paper was used for filtering under vacuum.

Preparation of Standard stock and Working solutions:-

Standard Stock solution of Betrixaban was prepared by dissolving 100 mg in 100 ml of methanol in volumetric flask to give a stock solution of 1000 µg/ml. The working standard solution was prepared by dilution of the stock solution with mobile phase Methanol: Water (80:20 v/v) to reach a concentration range 20 µg/mL for Betrixaban.

Selection of detection wavelength:-

Betrixaban standard stock solution was diluted appropriately and final concentration of solution obtained was 20 µg/mL. The spectrum was scanned for 20 µg/mL drug solution in the range from 200 to 400 nm and maximum wavelength (λ_{max}) obtained for Betrixaban was found to be 272 nm. UV-spectrum is shown in Fig. 2.

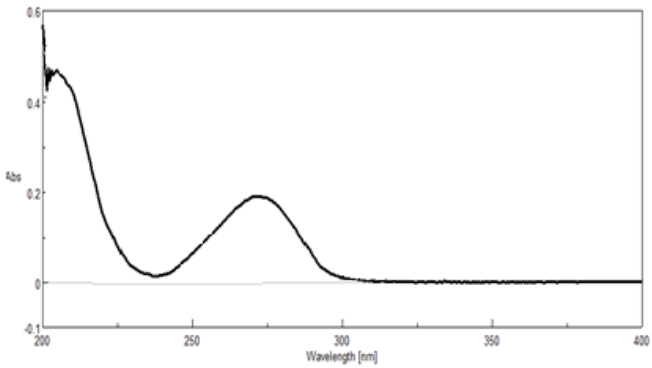

Fig. 2: UV spectrum of Betrixaban (10 µg/mL) in methanol

Forced Degradation Studies:

Forced degradation studies were carried out under alkali, acid, oxidation, neutral, photolytic and dry heat conditions. Two samples were prepared for each study. Betrixaban standard stock solution was prepared by weighing 100 mg of drug and transferring it to 100 mL volumetric flask and dissolving it in methanol to obtain a stock solution of 1000 µg/mL concentration.

Alkaline hydrolysis

From Betrixaban standard stock solution, 5 mL was mixed with 5 mL of 1 N NaOH. The mixture was allowed to stand at room temperature for 24 h. The solution was neutralized and further diluted upto 25 mL with methanol. From the above solution, 1 mL was withdrawn and diluted upto 10 mL with mobile phase (20 µg/mL) and was analyzed on HPLC.

ISSN (Online) 2456 -1304

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Vol 6, Issue 5, May 2021

Acid Hydrolysis

From Betrixaban standard stock solution, 5 mL was mixed with 5 mL of 1 N HCL. The mixture was allowed to stand at room temperature for 24 h. The solution was neutralized and further diluted upto 25 mL with methanol. From the above solution, 1 mL was withdrawn and diluted upto 10 mL with mobile phase (20 µg/mL) and was analyzed on HPLC.

Neutral Hydrolysis

From Betrixaban standard stock solution, 5 mL was mixed with 5 mL of distilled water. The mixture was allowed to stand at room temperature for 24 h and further diluted upto 25 mL with methanol. From the above solution, 1 mL was withdrawn and diluted upto 10 mL with mobile phase (20 µg/mL) and was analyzed on HPLC.

Oxidation

From Betrixaban standard stock solution, 5 mL was mixed with 5 mL of 6% H_2O_2. The mixture was allowed to stand at room temperature for 24 h and further diluted upto 25 mL with methanol. From the above solution, 1 mL was withdrawn and diluted upto 10 mL with mobile phase (20 µg/mL) and was analyzed on HPLC.

Degradation under dry heat

Drug sample was kept in oven at 60 ^oC for performing dry heat studies for duration of 4 h. Sample of quantity 10 mg was withdrawn after 24 h and transferred to 10 mL volumetric flask. The sample was dissolved in methanol and the volume was made up to 10 mL with methanol to get a stock solution of 1000 µg/mL. Further from 1000 µg/mL stock solution, 5 mL of solution was withdrawn and diluted to 25 mL with methanol to obtain working standard solution of 200 µg/mL. Further from 200 µg/mL solution, 1 mL was withdrawn and diluted upto 10 mL with mobile phase (20 µg/mL) and was analyzed on HPLC.

Photo-Degradation studies

Drug sample was exposure to direct sunlight for 12 h to perform photolytic studies. Sample of quantity 10 mg was withdrawn after 24 h and transferred to 10 mL volumetric flask. The sample was dissolved in methanol and the volume was made up to 10 mL with methanol to get a stock solution of 1000 µg/mL. Further from 1000 µg/mL stock solution, 5 mL of solution was withdrawn and diluted to 25 mL with methanol to obtain working standard solution of 200 µg/mL. Further from 200 µg/mL solution, 1 mL was withdrawn and diluted upto 10 mL with mobile phase (20 µg/mL) and was analyzed on HPLC.

Optimization of stability indicating HPLC method

To estimate Betrixaban, many different mobile phases were tried. The system suitability parameters were also taken into consideration like retention time (R_t), theoretical plates, tailing factor, good resolution between drug and degradation products. The optimized mobile phase was Methanol: Water in the ratio 80:20 v/v with a flow rate of 0.7 mL/min. Before injecting into HPLC system, the mobile phase was filtered using 0.45 µ filter paper to remove particulate matter and was degassed by sonication **(Fig. 3)**.

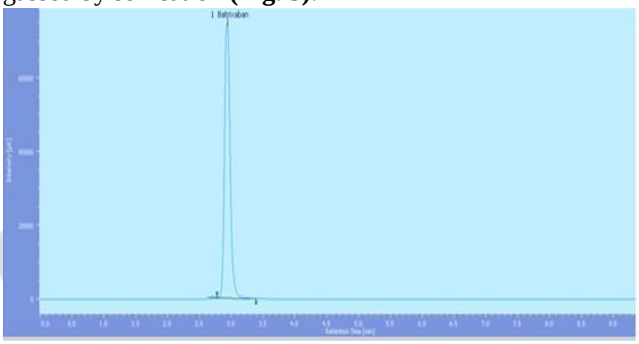

Fig 3: Chromatogram of standard Betrixaban 20 µg/mL

VALIDATION OF THE METHOD

The optimized LC method was validated as per the below parameters.

Linearity and range

The linearity of measurement was evaluated by analyzing nine different concentrations of the standard solution. Betrixaban was prepared in 10-90 µg/mL concentration range and was injected in HPLC system in triplicate. The injection volume was kept constant throughout analysis. The calibration graphs were plotted by taking peak areas against the corresponding concentrations.

Precision

Precision of the optimized method was carried out by performing repeatability and intermediate precision studies. Repeatability studies (intraday precision studies) were executed by analyzing three different concentrations of the drug i.e. 20, 60, 90 µg/ mL in hexaplicate on the same day. Intermediate studies (interday precision studies) was checked for the developed method by repeating studies on three different days.

Limit of detection and limit of quantitaiton

For estimating limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was injected six times following the same method as explained above. The signal to noise ratio (S/N) was determined. LOD and LOQ considered

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 6, Issue 5, May 2021

were 3:1 and 10:1. Betrixaban standard solutions were diluted until the average responses obtained were approximately 3 or 10 times the responses for six replicate of blank solution.

Robustness of the method

For evaluating robustness of the HPLC method, some parameters were deliberately changed. The parameters varied were flow rate, wavelength and pH of the mobile phase. Robustness studies were performed at three different levels i.e. -1, 0 and +1.

Specificity

The specificity of the developed method was evaluated by completely separating Betrixaban in presence of related impurities, degradation products, excipients.

Accuracy

For checking accuracy of the developed method, three different levels i.e. 80, 100, 120 % of standard drug was added to the sample. Samples chosen had a concentration of 20 µg/mL. The prepared solutions were injected in triplicate in optimized chromatographic conditions and % recovery was calculated.

Analysis of marketed formulation

The capsules of Betrixaban are not available in Indian market; hence in-house tablet was prepared. The capsules were prepared by using 80 mg of Betrixaban and 80 mg of excipient i.e, Opacifiers, Plasticizers, Preservatives, Sugar, Water and Sulfur Dioxide. The quantity of each ingredient was calculated & weighed for 20 capsules. These ingredients were mixed in a mortar in ascending order of their weight. Twenty capsules of Betrixaban were weighed, content was emptied and the average weight was calculated. The crystalline powder was crushed to furnish a homogeneous powder and quantity equivalent to one capsule was weighed in 100 ml volumetric flask. The lab formulation was sonicated with 10 ml of methanol for 5-10 min and volume was made up to 100 ml with methanol to get concentration 1000 µg/ml. The resulting solution was then filtered using 0.45µ Whatmann filter paper. The original stock solution was further diluted to get sample solution of drug concentration of 20 µg/ml for Betrixaban. Volume of 20 µL of sample solution was injected six times into RP-HPLC, under the above described conditions. The drugs were analyzed at 272 nm and samples concentrations were determined by means of multilevel calibration developed on the same RP-HPLC system under the same conditions using linear regression equation.

RESULTS AND DISCUSSION

Stability indicating property

HPLC studies of samples obtained on stress testing of Betrixaban under different conditions using methanol: water (80: 20 v/v) as a mobile phase suggested the following degradation behavior.

Alkali Hydrolysis

Initially, drug solution was kept for stress testing at room temperature for 24 h in 0.1 M sodium hydroxide but no degradation was witnessed. Hence, the strength of the base was increased. Further, studies were executed in 1 M sodium hydroxide. The drug showed 8.75 % degradation after exposing it for 24 h at room temperature.

Acid Hydrolysis

Initially, drug solution was kept for stress testing at room temperature for 24 h in 0.1 M hydrochloric acid but no degradation was witnessed. Hence, the strength of the acid was increased. Further, studies were executed in 1 M hydrochloric acid. The drug showed 7.42 % degradation after exposing it for 24 h at room temperature.

Neutral degradation

The drug solution was exposed to water for a duration of 24 h at room temperature, around 9.63 % of the drug showed degradation.

Oxidation

The drug solution was kept for stress testing at room temperature for 24 h with 6 % H_2O_2, around 4.43 % of degradation was observed.

Degradation under dry heat

Around 2.24 % of Betrixaban was found to degrade in thermal degradation, after exposing the drug to heat in oven at 60 ^oC for a period of 4 h.

Photochemical degradation

Betrixaban showed around 12.48 % of degradation in photochemical degradation, after exposing the drug to direct sunlight for 24 h.

Validation of the stability indicating method

The results of validation studies on the stability indicating method developed for Betrixaban in the current study involving methanol : water (80: 20) v/v as a mobile phase are given below.

Linearity

Betrixaban showed a linear response (R^2= 0.9998) in the

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Vol 6, Issue 5, May 2021

concentration range of 10-90 µg/mL. The mean (± RSD) value of slope was 8581.9 and value of intercept was 1989.1.

Precision

Table 1, 2, 3 depicts the results of repeatability and intermediate precision studies. As suggested in ICH guidelines, RSD values were < 2% for repeatability and intermediate precision studies. In stressed samples, separation of drug from its degradation products was found similar when analysis was performed on different chromatographic system on different days.

Table 1: Results of repeatability studies

Sr. No.	Amount Claimed (µg/ml)	Area	Amount found	%Amount found
1	20	173495	19.93	99.66
2	20	182965	20.02	100.10
3	20	172717	19.77	98.85
4	20	183246	20.15	100.76
5	20	173124	19.86	99.34
6	20	183198	20.13	100.69
Mean				99.9
Standard Deviation (S.D.)				0.7586
% Relative Standard Deviation (%RSD)				0.7593

SD and %RSD < 2 indicate that the proposed method is precise

Table 2: Results of Intra-day precision studies

Sr. No.	Amount claimed	Area	Amount found	%Amount found	Mean	SD	%RSD
1	20	173275	19.79	98.98	99.45	0.5335	0.5365
2	20	181761	20.01	100.03			
3	20	173821	19.86	99.34			
4	60	538986	59.54	99.23			
5	60	549674	60.21	100.36	99.66	0.6071	0.6091
6	60	539267	59.64	99.41			
7	90	829876	90.83	100.92	100.16	0.6653	0.6642
8	90	817695	89.91	99.90			
9	90	811243	89.70	99.67			

Table No. 3: Results of Inter-day precision studies

Sr. No.	Amount claimed	Area	Amount found	%Amount found	Mean	SD	%RSD
1	20	173245	19.77	98.85	99.64	0.7851	0.7879
2	20	181956	20.08	100.42			
3	20	179879	19.93	99.66			
4	60	548691	60.10	100.17	99.85	0.3883	0.3889
5	60	539271	59.65	99.42			
6	60	542378	59.98	99.97			
7	90	811021	89.67	99.63	99.27	0.6914	0.6964
8	90	810998	88.64	98.48			
9	90	812134	89.75	99.72			

SD and % RSD < 2 indicate that the proposed method is precise.

LOD and LOQ

The signal to noise ratios (S/N) of 3:1 was considered for LOD and 10:1 was considered for LOQ. The LOD was found to be 0.5980 µg/mL and LOQ was found to be 1.7381 µg/mL.

Robustness of the method

Each selected factor was changed at three levels i.e -1, 0

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 6, Issue 5, May 2021

and 1. One factor was changed at one time to evaluate the effect. Six replicate injections of standard solution at three concentration levels were performed by undergoing small changes in the chromatographic parameters or factors. Minor differences in peak areas and retention time were observed **(Table 4)**.

Table 4: Robustness testing (n = 3)

Factor	Level	Betrixaban (Retention Time)
A: Flow rate (ml/min)		
0.6	-0.1	2.89
0.7	0	2.91
0.8	+0.1	3.18
B: pH of mobile phase		
2.9	-0.1	2.8
3.0	0	2.91
3.1	+0.1	3.09
C: Wavelength change		
271	-0.1	2.81
272	0	2.91
273	+0.1	3.2

Specificity

The specificity of the HPLC method is evident from the separation of Betrixaban in presence of its degradation products. Sharp peaks were obtained with clear baseline separation. The resolution obtained between the nearest peak was more than 2. The detector used was PDA, which scanned the components in the wavelength ranging from 200 to 400 nm and indicated that there is no unresolved peak (of blank/degradant/impurity/placebo) from the pure drug. Also the peak purity of analyte peak was found to be more than 0.999.

Recovery studies

From **Table 5,** it is evident that good recoveries of the drug are obtained in the range from 98.30 to 100.46 % at various added concentrations, in spite of the fact that the drug was exposed to degradation product formed at various reaction conditions.

Table 5: Recovery studies (n = 6)

Level	Conc. (µg/mL)		Area	% Recovery
	Sample	Std.		
80%	20	16	315674	99.53
	20	16	321675	100.46
	20	16	321465	99.84
100%	20	20	354367	98.30
	20	20	362415	99.94
	20	20	361284	99.86
120%	20	24	383594	100.07
	20	24	382415	99.65
	20	24	376415	99.39

Level of recovery (%)	Mean	SD	%RSD
80%	99.94	0.4735	0.4768
100%	99.36	0.9246	0.9310
120%	99.70	0.3431	0.3455

Analysis of marketed formulation

The drug content was found to be 99.77 % ± 0.66 and the results are summarized in **Table 6**.

Table 6: Analysis of commercial formulation

Sr. No.	Amount taken (µg/ml)	Peak Area	Amount of drug found (µg/ml)	% Amount found
1	20	179912	19.94	99.72
2	20	181898	20.01	100.03
3	20	173346	19.79	98.98
4	20	182101	20.09	100.49
5	20	173314	19.80	99.02
6	20	181956	20.08	100.42
Mean				99.77
Standard Deviation (S.D)				0.6629
%Relative Standard Deviation (%R.S.D)				0.6644

CONCLUSION

A stability indicating RP-HPLC method is developed and validated to estimate Betrixaban in pure and capsules dosage form. The column used was Hi Q Sil C_{18} (250 mm × 4.6 mm), 5 µ as stationary phase. After trying several permutation and combinations, Methanol: Water in the ratio of (80:20 v/v) gave good resolution between peaks, with acceptable peak symmetry, theoretical plates as compared to other mobile phases. The optimized flow rate for the method was 0.7 mL/min. Solutions of Betrixaban in appropriate dilution were scanned using Jasco UV-Visible spectrophotometer V-630 in the spectrum mode from the wavelength range of 400 nm to 200 nm. The wavelength selected was 272 nm as the drug was found to have significant absorbance at this wavelength. Linearity was observed in the range of 10-90 µg/ml for Betrixaban. The retention time for Betrixaban was found to be 2.91 min respectively. The correlation coefficient for the drug was found to be in the range of 0.9998. It is observed that the Betrixaban has shown 8.75 % degradation in alkaline

medium, 7.42 % degradation in acidic medium, 9.63 % degradation in water, 4.43 % degradation in hydrogen peroxide, 2.24 % degradation in dry heat and 12.48 % degradation in photochemical degradation study.

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