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Development and validation of a stability-indicating RP-HPLC method for the determination of Betrixaban in bulk and its laboratory synthetic mixer.

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ABSTRACT

A stability-indicating reversed-phase liquid chromatography method (RP-HPLC) was validated for the analysis of Betrixaban. Analysis was performed on a Waters Alliance 2695 separation module, with waters 2487 UV detector in isocratic mode using Auto sampler. Data collection and processing was done using EMPOWER software version-2. The analytical column used for the separation was Xterra C_{18} (4.6 x 150mm, 5.0µm) , Flow rate was kept at 0.6 ml/min. the column temperature was maintained at Ambient. the mobile phase was made up of Orthophosphoric acid (0.1%) pH 2.1 and Methanol in 10:90 ratio. The method was optimized at 292nm. Run time was taken as 10 min. The injection volume of samples was 20 µl.The separation was obtained with retention time of 2.663 min, and was linear over the concentration range of 100-600 ppm (r2 = 0.999). The specificity and stability-indicating capability of the method were proven through degradation studies. The LOD and LOQ of Betrixaban was found to be 3.2 µg/ml and 9.6 µg/ml respectively and the statistics data for the Betrixaban was concluded that the method was found to be simple, reliable, selective, reproducible and accurate. The method was successfully used for quality control analysis of Betrixaban.

Keywords: Betrixaban, RP-HPLC, Stability, and Validation.

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INTRODUCTION

Betrixaban [1] is chemically described as N-(5-chloropyridin-2-yl)-2 [4-(N,N-dimethylcarbamimidoyl)-benzoylamino]-5-methoxybenzamidemaleate. Its molecular formula (as maleate salt) is $C_{27}H_{26}ClN_5O_7$, which corresponds to a molecular weight of 567.98 mg . Betrixaban is an oral FXa inhibitor that selectively blocks the active site of FXa and does not require a cofactor (such as Anti-thrombin III) for activity. Betrixaban inhibits free FXa and prothrombinase activity. By directly inhibiting FXa, betrixaban decreases thrombin generation (TG). Betrixaban has no direct effect on platelet aggregation. Betrixaban Is a one of the newer tablet dosage form which is used to treat the platelet aggregation.author made an attempt to Develop a stability-indicating RP-HPLC method for the determination of Betrixaban in bulk and its laboratory synthetic mixer [2-6]. Chemical structure of Betrixaban is shown in Figure No. 1.

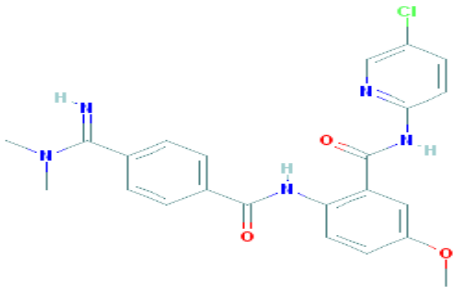

Figure 1: Chemical structure of Betrixaban

MATERIALS AND METHODS

Chemicals and reagents:

Betrixaban was obtained as gift sample pharmatrain labs, Hyderabad, India. Methanol (HPLC Grade; Fischer), Water (HPLC Grade; Fischer), Acetonitrile (HPLC Grade; Fischer), Ortho phosphoric acid (G.R; Fischer), 0. 22µ PDFA filter(HPLC Grade; Advanced lab), 0.45µ filter paper(HPLC Grade; Millipore) were used for the entire study.

Preparation of standard solutions for HPLC:

Preparation of OPA buffer:

1ML Of OPA was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water and pH was adjusted to 3 with NAOH . The resulting solution was sonicated and filtered.

Preparation of mobile phase:

Mix a mixture of buffer 100 ml (10%) and 900 ml Methanol HPLC (90%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Diluents preparation:

Mobile phase was used as the diluent. 0.1% OPA : Methanol (10:90) ratio

Preparation of the Betrixaban standard preparation:

Accurately weigh and transfer 10 mg of Betrixaban is taken into a 10ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the diluent. (Stock

solution)Further pipette 3.0 ml of Betrixaban of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

In-House tablet preparation of Betrixaban:

40 mg of standard drug and microcrystalline cellulose as a diluent were weighed accurately and co-sifted by passing through #40 sieve, blended in a poly bag for 5min. The above blend were lubricated with #60 sieve passed Talc and magnesium stearate. The final blend was then compressed in to tablets using 16 station compression machine with an average hardness of 4-6kg/cm^2.

Sample solution preparation:

Accurately weigh and transfer equivalent to 10mg of Betrixaban sample is taken into a 10ml clean dry volumetric flask add diluents and sonicate to dissolve it completely and make volume up to the mark with the diluent. (Stock solution) Further pipette 3.0 ml of Betrixabanof the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

HPLC Instrumentation and chromatographic condition:

Analysis was performed on a Waters Alliance 2695 separation module, with waters 2487 UV detector in isocratic mode using Auto sampler. Data collection and processing was done using EMPOWER software version-2. The analytical column used for the separation was Xterra C $_{18}$ (4.6 x 150mm, 5.0µm) , Flow rate was kept at 0.6 ml/min. the column temperature was maintained at Ambient. the mobile phase was made up of Orthophosphoric acid (0.1%) pH 2.1 and Methanol in 10:90 ratio. The method was optimized at 292nm. Run time was taken as 10 min. The injection volume of samples was 20 µl.

Other equipment's used were ultra-sonicator (model SE60US, Enertech), Digital weighing balance(sensitivity 5mg) (model ER 200A Ascoset), pH meter (model AD 102U , ADWA). The retention times for **Betrixaban** was found to be 2.663 mins. Typical chromatogram of **Betrixaban** show in Figure No. 2 and optimized chromatographic conditions for proposed method details are shown in Table No 1.

Table 1: Instrumentation and Optimized chromatographic conditions for proposed method

S.No	Instrumentation	Optimized chromatographic conditions
1	Column	Xterra C $_{18}$ (4.6 x 150mm, 5.0µm)
2	Mobile phase	0.1% OPA: Methanol (10:90)
3	Flow rate	0.6 mL per min
4	Wavelength	292 nm
5	Injection volume	20 µl
6	Column temperature	Ambient
7	Run time	10 min
8	Retention time	2.663 min

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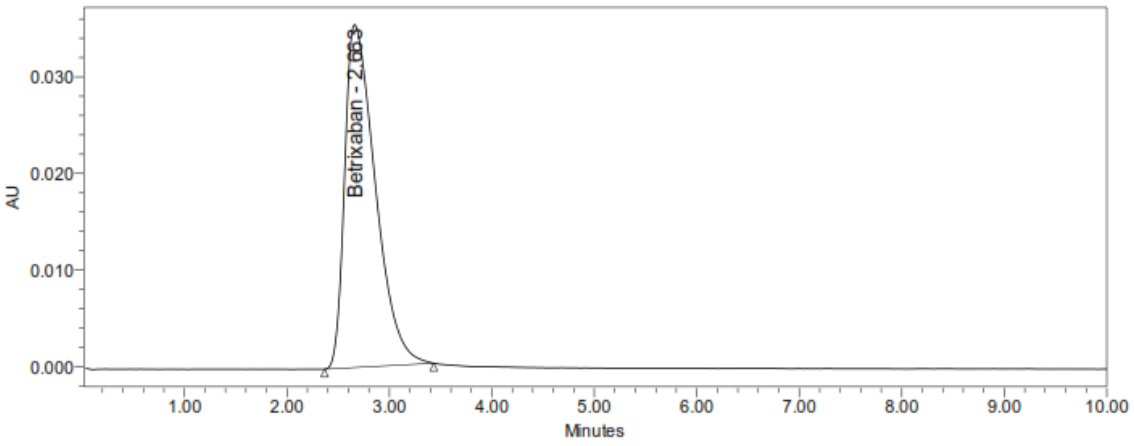

Figure 2: RP-HPLC Optimized chromatogram of Betrixaban

RESULTS AND DISCUSSION

Validation study of Betrixaban

The Method validation was performed as per ICH guidelines for the simultaneous estimation of Betrixaban in bulk and pharmaceutical dosage form. The method was validated with respect to parameters including accuracy, precision, linearity, robustness, specificity, system suitability, LOD and LOQ.

Assay of Betrixaban

The developed method was applied to the assay of Betrixaban in pharmaceutical formulation. The drug content was estimated with an average of three determinations, and results were given in Table No 2.

Table 2: Linearity and sensitivity data of the proposed method

Parameter	Betrixaban
Linearity (ppm)	100 ppm - 600ppm
Regression equation (y* = m x** + c)	y = 2368.4x – 2426
Slope (m)	2368.4
Intercept (c)	2426
Correlation coefficient (R 2)	0.9999
LOD (µg/mL)	3.2
LOQ (µg/mL)	9.6
Assay	99.74%

Linearity

The linearity study was performed for the concentration of 100 ppm to 600ppm Betrixaban level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. The results were shown in Table No 2.calibration graph was shown in graph no 1. The results demonstrate an excellent correlation between the peak area and concentration of analytes in the concentration range of 100 ppm to 600ppm Betrixaban.

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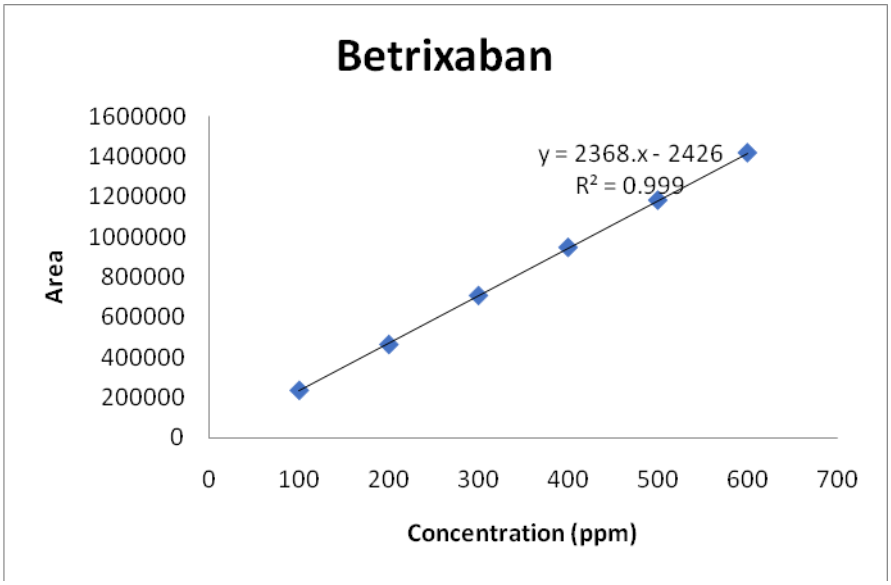

Graph 1: Linearity Graph of Betrixaban

Detection limit

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines and results were given in Table No 2.

Quantitation limit

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines and results were given in Table No 2.

Accuracy

The accuracy study was performed for 50%, 100% and 150 % for Betrixaban. Each level was injected in triplicate into chromatographic system. The area of each level was used f or calculation of % recovery. Recovery data for proposed method are shown in Table No 3.

Table 3: Recovery data for the proposed RP-HPLC method for Betrixaban

(at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	356603.3	5	5	100.05	100.19
100%	715316.7	10	10.03	100.35	
150%	1071110.0	15	15.03	100.17	

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Precision

Repeatability

The precision study was performed for six injections of Betrixaban. Each standard injection was injected into chromatographic system.The area of each Standard injection was used for calculation of % RSD and results are tabulated in Table.No.4

Table 4: Precision results of the proposed RP-HPLC method

System precision			Method precision		
Betrixaban					
Amount of drug (µg/mL)	**Peak area**	**Statistical Analysis**	**Amount of drug (µg/mL)**	**Peak area**	**Statistical Analysis**
300	719214	**Mean:** 715683.7 **SD:** 2264.1 **%RSD:** 0.3	300	719651	**Mean:** 715761.7 **SD:** 3322.2 **%RSD:** 0.5
300	712824		300	712440	
300	716267		300	712044	
300	716618		300	719227	
300	715421		300	714350	
300	713758		300	716858	

Intermediate precision/Ruggedness

The intermediate precision study was performed for five injections of Betrixaban. Each standard injection was injected into chromatogaphic system. The area of each standard injection was used for calculation of % RSD.

Robustness

The robustness was performed for the flow rate variations from 0.63 ml/min to 0.77 ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Betrixaban .The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±10% RSD and results are tabulated in Table.No.5

Table 5: Robustness results of the proposed RP-HPLC method for Betrixaban

S. No	Parameters		USP	
			Plate Count	**Tailing**
1	Flow rate	0.54	3132.20	1.21
		0.60	2892.94	1.13
		0.66	3167.78	1.21
2	Mobile phase composition	10 % less	3039.17	1.09
		90:10	2892.94	1.13
		10 % more	3879.34	1.22

DEGRADATION STUDIES

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Betrixaban using the proposed method. Results are tabulated in Table.No.6 and chromatograms are show in fig no 3.

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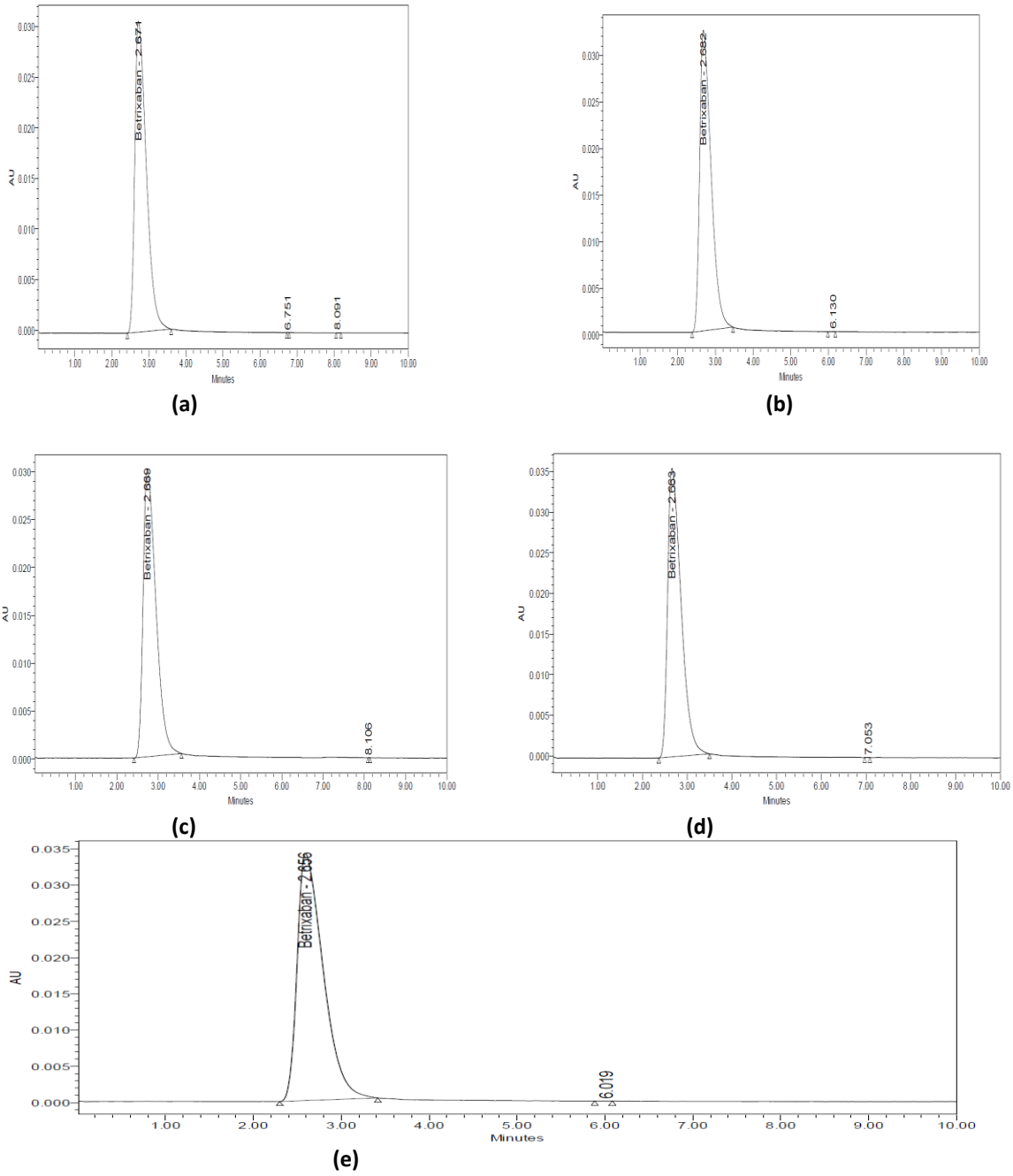

Figure 3 (a-e): chromatograms of degradation-(a) acid degradation (b) Alkali degradation (c) peroxide degradation (d) photo degradation (e) thermal degradation sample.

Table 6: Degradation results of the proposed RP-HPLC method for Betrixaban

Sample Name	Betrixaban	
	Area	% Degraded
Standard	711402.7	
Acid	661616	7.00
Base	675297	5.08
Peroxide	644593	9.39
Thermal	697906	1.90
Photo	646473	9.13

Preparation of stock:

Take the dry and clean 25ml volumetric flask, and then weighed accurately 25 mg of Betrixaban into the 25ml volumetric flask and made up to the mark with diluent to get stock solution 1000µg/ml this solution subjected to the sonication.

Hydrolytic degradation under acidic condition:

Pipette 3 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials. 10µl solution were injected in to the system and the chromatograms were recorded to assess the stability of sample.

Hydrolytic degradation under alkaline condition:

Pipette 3ml of above solution into a 10ml volumetric and add 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials. 10µl solution were injected in to the system and the chromatograms were recorded to assess the stability of sample.

Thermal induced degradation:

Betrixaban sample was taken in petridish and kept in Hot air oven at 110^0 C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Oxidative degradation:

Pipette 3ml above stock solution into a 10ml volumetric flask and 1ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials. 10µl solution were injected in to the system and the chromatograms were recorded to assess the stability of sample.

Photo degradation:

Pipette 3 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24hrs and the volume was made up to the mark with diluent. Filter the solution with 0.45 microns syringe filters and place in vials and injected into HPLC and analysed.

CONCLUSION

A new method was established for simultaneous estimation of Betrixaban by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Betrixaban by using Xterra C_{18} (4.6 x 150mm, 5.0µm), flow rate was 0.7ml/min, mobile phase ratio was (90:10 v/v) Methanol: 0.1 % OPA buffer pH 3.5 (pH was adjusted with NAOH), detection wave length was 292 nm The instrument used was WATERS HPLC Auto Sampler, Waters 486, tunable absorbance detector, Empower-software version-2. The retention time was found to be 2.160 . The % purity of Betrixaban was found to be 99.74% respectively. The system suitability parameters for Betrixaban such as theoretical plates and tailing factor were found to be 2179.10, 1.19. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study of Betrixaban was found in concentration range of 100µg-600µg and correlation coefficient (r^2) was found to be 0.999 , % recovery was found to be 100.19% , %RSD for repeatability was 0.3, % RSD for intermediate precision was 0.5 respectively. The precision study was precision, robustness and repeatabilty.LOD value was 3.2 and LOQ value was 9.6 respectively.

Hence the suggested RP-HPLC method can be used for routine analysis of Betriaxaban in API and Pharmaceutical dosage form.

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