EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 8
372-382
Year: 2018

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF BOSENTAN MONOHYDRATE IN BULK AND TABLET DOSAGE FORM

Rutuja Prabhakar Sonare*, Dr. Ashok Pandurang Pingle, Komal Ashok Rajgire, Rina Mohan Sonawane and Snehal Ganpat Tekawade

Maratha Vdiya Prasarak Samaj's College of Pharmacy Nasik Department of Pharmaceutical Chemistry.

***Corresponding Author: Rutuja Prabhakar Sonare**
Department of Pharmaceutical Chemistry, Maratha Vidya Prasarak Samaj's College of Pharmacy Nashik.

Article Received on 14/06/2018 **Article Revised on 04/07/2018** **Article Accepted on 24/07/2018**

ABSTRACT
A simple, rapid, economic, sensitive and precise Stability indicating RP-HPLC method has been developed for the determination of Bosentan Monohydrate in bulk and pharmaceutical dosage form. The method is carried out using Grace C18 (4.6ID×250mm; 5µm) column and mobile phase comprised of Methanol: Potassium dihydrogen phosphate buffer in proportion of ratio 90:10v/v and degassed under ultrasonication. The flow rate was 0.9ml/min and detection was carried out at 222nm. The retention time of Bosentan Monohydrate were found to be 4.963min. The method was validated in terms of linearity, precision, accuracy, limit of detection, limit of quantitation, robustness and ruggedness. The method was also applied for the determination of Bosentan Monohydrate in presence of their degradation products formed under variety of stressed conditions. Degradation product produce as a result of stress studies did not interfere with the Bosentan Monohydrate and the assay can thus be considered stability indicating. The proposed method is suitable for the routine quality control analysis for determination of Bosentan Monohydrate in bulk and pharmaceutical dosage form.

KEYWORDS: Bosentan Monohydrate, RP-HPLC, stability indicating, stress studies, validation.

1. INTRODUCTION
[1]Pulmonary arterial hypertension (PAH) is a chronic, life-threatening disorder which severely hampers the function of the lungs and heart. In individuals with PAH, the levels of endothelin which is a potent endogenous vasoconstrictor is increased. Bosentan Monohydrate (BSN) has been prescribed for the management of PAH. BSN is a vasodilator and oral dual endothelin receptor antagonist. BSN acts by competitively antagonizing the binding of endothelin to both endothelin receptors, ET-A and ET-B. Chemically Bosentan Monohydrate is 4-tertbutyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxy-Phenoxy)-2(Pyrimidin-2-yl) pyrimidin-4-yl] benzene - 1-sulphonamide monohydrate[1] (figure 1).

[2]Bosentan is a dual endothelin receptor antagonist important in the treatment of pulmonary arterial hypertension (PAH). Bosentan is used to treat pulmonary hypertension by blocking the action of endothelin molecules that would otherwise promote narrowing of the blood vessels and lead to high blood pressure.[2]

[3]Endothelin-1 (ET-1) is a neurohormone, the effects of which are mediated by binding to ET-A and ET-B receptors in the endothelium and vascular smooth muscle. ET-1 concentrations are elevated in plasma and lung tissue of patients with pulmonary arterial hypertension, suggesting a pathogenic role for ET-1 in this disease. Bosentan is a specific and competitive antagonist at endothelin receptor types ET-A and ET-B. Bosentan has a slightly higher affinity for ET-A receptors than for ET-B receptors.[3]

Bosentan belongs to a class of drugs known as endothelin receptor antagonists (ERAs). Bosentan blocks the binding of endothelin to its receptors, there by negating endothelin's deleterious effects. It is manufactured in India by Lupin and Cipla Pharmaceuticals under the brand name of Lupibose and Bosentas respectively. Several analytical methods have been described in the literature for the determination of Bosentan in pharmaceutical dosage forms and biological fluids.[2]

There are few reports on the application of HPLC with UV detection for the assay of BSN in bulk drug and pharmaceutical dosage forms. The disadvantages of the reported HPLC with UV detection methods are lack of sensitivity, lesser precision and having a narrow range of linear response. In addition, the retention time of the

BSN is more which leads to a longer runtime for a single sample. The aim of the present study is to develop and validate a simple, fast, sensitive and precise stability indicating RP-HPLC method for the assay of BSN in bulk and in pharmaceutical dosage forms.[1]

Structure

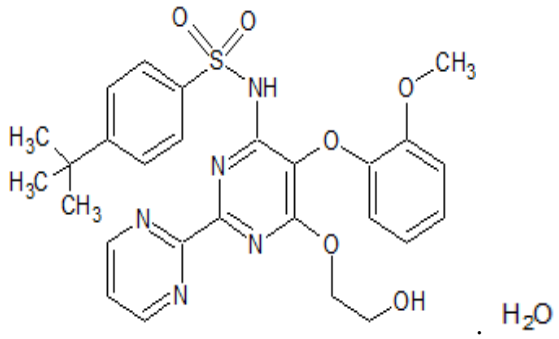


Fig. 1: Chemical structure of Bosentan monohydrate.

2. MATERIALS AND METHOD

2.1 Chemical and reagents

Bosentan was obtained as gift sample from, pure chem. Pvt. Ltd. Gujarat, India. The purity of the drug was evaluated by obtaining its melting point and ultraviolet (UV) and infrared (IR) spectra. No impurities were found. The drug was used without further purification.

HPLC-grade Methanol, Water and other reagents were of standard Quality. Potassium Dihydrogen Phosphate (AR grade) was obtained from Merck. A tablet formulation of Bosentan (62.5mg) was procured from local market.

2.2 Instrumentation

The chromatographic technique was performed on Shimadzu UV 2450 Double beam UV-Visible spectrophotometer with software UV probe, reversed phase Grace C18 column (4.6ID×250mm; 5µm) as stationary phase, Ultrasonic cleaner, Wenser High Precision Balance PGB 100, Wenser Ultra Sonicator WUC- 4L, Vacuum micro filtration unit with 0.45µ membrane filter was used in the study.

2.3 Chromatographic condition

Chromatographic separation was performed on Grace C18 analytical column. Isocratic mobile phase consisting of Methanol: Potassium diphosphate buffer (90:10, v/v) was delivered at flow rate 0.9ml/min. injection volume was 20µL.

2.4 Preparation of calibration standards for UV

Standard stock solution(A_1): An accurately weighed about 10.0mg quantity of Bosentan monohydrate was transferred in a 10.0mL of volumetric flask, dissolved in a sufficient quantity of methanol and volume was made up to the mark with methanol. (Conc. 1000µg/ml of Bosentan monohydrate).

Working stock solution (A_2): A 1.0mL of standard stock solution was transferred in 10.0mL of volumetric flask and volume was made up to the mark with methanol. (Conc. 100µg/ml of Bosentan monohydrate).

Working standard solution: A 0.3mL of working stock solution (A_2) was transferred in 10.0mL of volumetric flask and volume was made up to the mark with methanol (Conc. 3µg/ml Bosentan Monohydrate). Then further dilution of drug like 6, 9, 12, 15µg/ml from stock solution was prepared in the same solvent.

Selection of wavelength: Standard solution of 3-15µg/ml was scanned between 400nm to 200nm.

2.5 Sample Preparation

Standard stock solution: An accurately weighed about 10.0mg Bosentan Monohydrate was transferred in a 10.0mL volumetric flask, dissolved in sufficient quantity and volume was made up to the mark with mobile phase (Conc. 1000µg/ml).

Working standard solution: A 0.1mL of stock solution (A1) was transferred in 10.0mL volumetric flask and volume was made up to the mark with mobile phase (Conc. 10µg/ml). Then further dilution of drug like 20, 30, 40, 50µg/ml from stock solution was prepared in the same solvent.

2.6 Potassium Dihydrogen Phosphate Solution (10mM)

10mM solution may be prepared by dissolving 0.136g of potassium dihydrogen phosphate in sufficient water then volume make up to 100ml. pH adjusted with OPA (ortho-phosphoric acid).

2.7 Method Validation

The method was validated for the following parameters: linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, robustness and ruggedness.

A. Linearity

Linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range.[4]

Linearity was performed by diluting standard stock solution to give final concentration in the range of 10µg/ml to 50µg/ml for Bosentan Monohydrate. 20µL of each concentration injected and calibration curve was constructed by plotting the peak area versus the drug concentration. Correlation coefficient should not be less than 0.999.

B. Accuracy

[4]The accuracy of an analytical method is closeness of test results obtained by that method to the true value [4]. Accuracy is calculated from the test result as the percentage of analyte recovered by the assay. Accuracy was performed in triplicates and compares the results. % recovery was performed by spiked known quantity of

drug at 50%, 100% and 150% to a pre-quantified sample solution and analyses sample. From the result % recovery was calculated.

1. Mean recovery should be in the range of 98-102%
2. The relative standard deviation should not be more than 2.0%

C. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.[4]

Prepare six different test solutions. Inject duplicate injection of each test solution. Pipette 0.3ml of stock solution in 10ml volumetric flask and dilute with solvent in sufficient quantity and make volume up to mark with solvent to get concentration of Bosentan monohydrate 30ppm.

D. Limit of Detection (LOD)[4]

The lowest concentration of the analyte in the sample that the method can detect but not necessarily quantify under the stated experimental conditions simply indicates that the sample is below or above certain level. Limit test prescribed as percentage or as parts per million. It can be calculated as:

$$LOD = \frac{3.3 \times SD}{S}$$

Where, SD=standard deviation, S=slope.

E. Limit of Quantitation(LOQ)[4]

The limit of quantitation is the lowest amount of analyte in the sample that can be determined with acceptable precision & accuracy under the stated experimental conditions. It is expressed as the conc. of analyte in sample. It can be calculated as:

$$LOQ = \frac{10 \times SD}{S}$$

Where, SD=standard deviation, S=slope.

F. Robustness

[4]It is the measure of capacity of the method to remain unaffected by small but deliberate change in the method parameter and provide an indication of its reliability under normal usage.[4] The robustness of an analytical method is determined by analysis of aliquots from homogenous lots by differing physical parameters that may differ but are still within the specified parameters of the assay. Carry out the following procedure individually by changing in pH 2.6 and 3.4 and flow rate 0.7 and 1.1ml/min.

G. Ruggedness

The ruggedness of analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different instruments, different lots of reagents, different assay, temperatures, different days, different analysts, etc.

The ruggedness of an analytical method is determined by analysis of aliquots from homogenous lots by different analyst, and instrument that may differ but are still within the specified parameters of the assay.

H. Analysis of marketed tablet formulation (Assay)

Twenty tablets of each brand (Bosentas) were purchased from the local market, weighed and crushed to a fine powder. Accurately weigh and transfer a quantity of powder sample equivalent to 10 mg of Bosentan Monohydrate into a 10ml clean volumetric flask, add sufficient diluents and sonicate to dissolve it completely and make volume up to the mark with the diluents. Filter the solution through 0.45µm membrane filter. Pipette out 0.3 ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents for 30µg/ml solution.

I. Force degradation study[2,5,7]

Forced degradation studies are also known as stress testing, stress studies, stress decomposition studies, forced degradation studies etc. forced degradation is a process that involves degradation of drug products and drug substances at conditions more severe than accelerated conditions and thus generates degradation products that can be studied to determine the stability of the molecule.

Drug sample were exposed to the following different stress conditions.

- **Acid degradation sample:** Accurately weigh and 10mg of Bosentan Monohydrate drug powdered into a 10ml clean dry volumetric flask, add sufficient amount of diluents and sonicate to dissolve it for about 10 min with intermediate shaking at controlled temperature. Then add 5ml of 1N acid (HCL), refluxed for 60 minutes at 60°C, then cooled to room temperature, neutralize with 5ml of 1N base (NaOH) and make volume up to the mark with diluents and mix. Filter the solution through 0.45um membrane filter. Further pipette 0.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

- **Base degradation sample:** Accurately weigh and 10mg of Bosentan Monohydrate drug powdered into a 10ml clean dry volumetric flask, add sufficient amount of diluents and sonicate to dissolve it for about 10 min with intermediate shaking at controlled temperature. Then add 5ml of 1N base (NaOH), refluxed for 60 minutes at 60°C, then cooled to room temperature, neutralize with 5ml of 1N acid (HCL), and make volume up to the mark with diluents and mix. Further pipette 0.5ml of the above stock

solution into a 10ml volumetric flask and dilute up to the mark with diluents.

- **Oxidative degradation sample:** Accurately weigh and 10mg of Bosentan Monohydrate drug powdered into a 10ml clean dry volumetric flask, add sufficient amount of diluents and sonicate to dissolve it for about 10 min with intermediate shaking at controlled temperature. Then add 5ml of 3% peroxide, refluxed for 60 minutes at 60°C, then cooled to room temperature, neutralize with 5ml of 1N acid (HCL), and make volume up to the mark with diluents and mix. Further pipette 0.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

- **Photolytic degradation sample:** The drug was exposed to UV cabinet for about 24hr. accurately weigh and transfer 10mg of Bosentan Monohydrate drug powder into a 10ml clean dry volumetric flask, add sufficient amount of diluents and sonicate to dissolve it for about 10 min with intermediate shaking at controlled temperature. Then make volume up to the mark with the diluents. Further pipette 0.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Acceptance criteria

1. Degradation should not be more than 20%
2. Degradation products should be resolved from the drug peak.

3 RESULT AND DISCUSSION

The present work was aimed to develop a stability-indicating RP-HPLC method for the determination of Bosentan Monohydrate in its tablet formulations in presence of its degradants. A reversed-phase chromatographic technique was developed to determine Bosentan Monohydrate at 222 nm (fig.2) and Grace C18 column was adopted for the analysis.

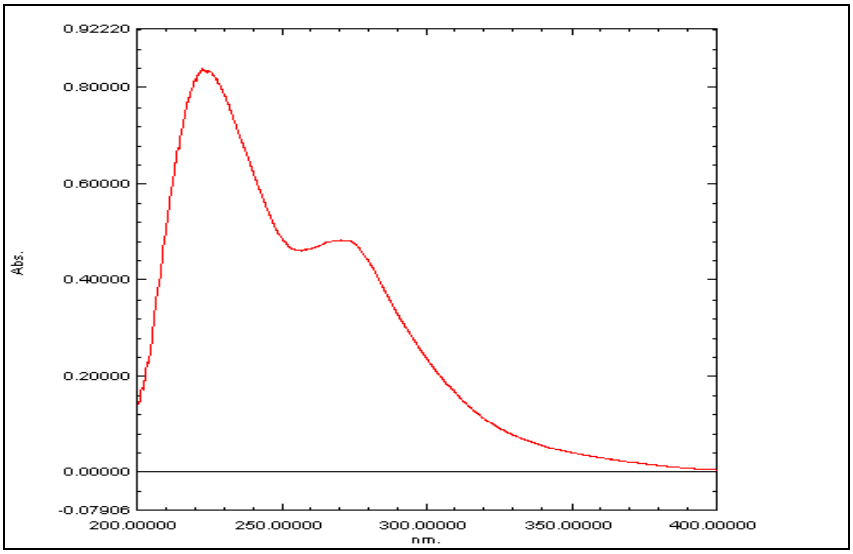


Fig. 2: Absorption spectrum of Bosentan Monohydrate (15µg/ml).

RP-HPLC method development and optimization: In the literature survey of Bosentan Monohydrate no suitable stability-indicating assay method was available for the determination of Bosentan Monohydrate in presence of its degradation products using RP-HPLC. A mixture of Methanol: Potassium dihydrogen phosphate was selected as mobile phase. Initially the Bosentan Monohydrate drug sample solutions were analyzed using a mobile phase consisting of Methanol: Water (80:20, v/v) at a flow rate of 0.8 ml/min. Under these conditions, a broad peak was observed at 7.368 min. Then the mobile phase was changed to Methanol: Potassium dihydrogen phosphate buffer 80:20, v/v and 90:10, v/v with a flow rate of 0.8 ml/min and 0.9 ml/min resp. and as a result retention time was 7.353 and 4.963min resp. So the flow rate was totally changed to 0.9 ml/min under which a sharp peak was eluted with good symmetry and retention time 4.963 ± 0.46 min. Hence a mobile phase containing Methanol: Potassium dihydrogen phosphate (90:10, v/v) with flow rate of 0.9 ml/min was chosen as the best chromatographic condition for the entire study.

Method validation

A. Linearity

The calibration curve for Bosentan Monohydrate was linear over the concentration range 10-50µg/ml. Calibration curve was constructed by plotting the peak area versus the drug concentration. The data for the peak area of the drug corresponding to the concentration was treated by linear regression analysis (Table 1 and fig. 3) and the regression equation for the calibration curve was found to be y = 78742x + 268970 with correlation coefficient of 0.9996 which is nearly equals to unity.

Table No. 1: Linearity of Bosentan Monohydrate.

Sr.no	Concentration (µg/ml)	Area
1	10	1024623
2	20	1872188
3	30	2650075
4	40	3422938
5	50	4186360
Correlation coefficient	0.9996	
Slope(m)	78742	
Intercept(c)	268970	

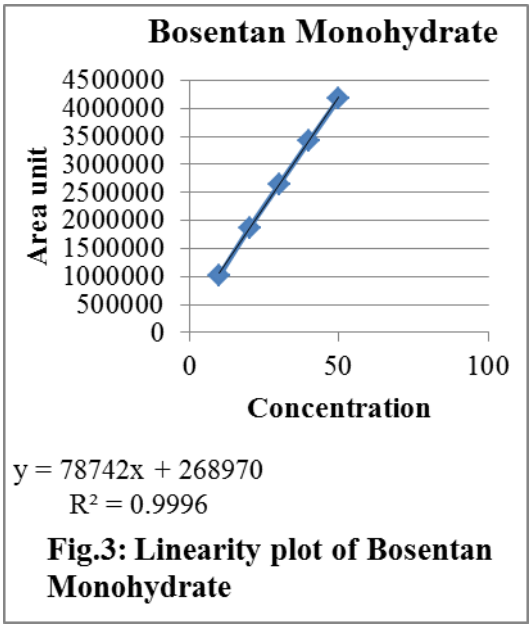


Fig.3: Linearity plot of Bosentan Monohydrate

B. Accuracy

Accuracy was performed in three different levels for Bosentan Monohydrate. Analysis was done in triplicate for each leel. Accuracy results were expressed in terms of percent recovery.

The resultant % RSD was in the range 0.08-0.15 (<2.0%) with recovery 99.82-99.92% (Table 2 and 3).

Table No. 2: Accuracy study of Bosentan Monohydrate.

			Standard Deviation		Accuracy	Precision
Sr. no.	Conc.	Area	Mean	SD	%SD	%RSD
	10	1024623	1025539.333	868.7418105	0.08471073	0.084710726
1	10	1026351				
	10	1025644				
	30	2650075	2653632.667	4234.934986	0.1595901	0.159590098
2	30	2652506				
	30	2658317				
	50	4186360	4188453.333	2295.429662	0.05480375	0.054803754
3	50	4188092				
	50	4190908				

Table No. 3: % recovery of Bosentan Monohydrate.

Sr. No.	% Composition	Standard sample	Tablet sample	Area of Standard	Area of Sample	% Recovery
1	**50% Recovery**	20ppm	10ppm	2650075	2646336	99.85890965
2	**100% Recovery**	20ppm	20ppm	3422938	3420224	99.92071139
3	**150% Recovery**	20ppm	30ppm	4186360	4179057	99.82555251

C. Precision

The precision of the method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision). Repeatability was calculated by assaying three samples of each at three concentration levels (30µg/ml) on the same day. The inter-day precision was calculated by assaying three samples of each at three concentration levels (30µg/ml) on two different days. The % RSD in precision studies was found to be 0.14% (intra-day) and 0.17% (Interday) which is <2.0% (Table 4).

Table No. 4: Precision studies of Bosentan Monohydrate.

Intraday				
Concentration	**Area**		**Mean**	**% RSD**
30 µg/ml	**Morning**	**Evening**	2652397	0.14%
	2650007	2652762		
	2652506	2646887		
	2658317	2653837		
Interday				
Concentration	**Area**		Mean	% RSD
30 µg/ml	**Day 1**	**Day 2**	2649054	0.17%
	2650007	2648009		
	2652506	2645068		
	2658317	2649054		

D. Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD was found to be 0.1033µg/ml and LOQ was found to be 0.3132µg/ml.

E. Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate change in method parameter and provide an indication of its reliability for routine analysis. The robustness of the method was evaluated by assaying the same sample under different analytical conditions deliberately changing from the original condition. The detection wavelength was set at 222nm, the ratio of methanol: Potassium Dihydrogen Phosphate in the mobile phase was applied as 90:10 v/v. The flow rate was set at 0.7 and 1.1 ml/min (± 0.2 ml/min) and pH was set at 2.6 and 3.4(±04). The results obtained (Table 5) from assay of the test solutions were not affected by varying the conditions and were in accordance with the results for original conditions. The %SD value of assay determined for the same sample under original conditions and robustness conditions was less than 2.0% (0.06-0.10) indicating that the method is robust (Table 5).

Table No. 5: Robustness study of Bosentan Monohydrate.

Sr.no.	Parameter		Conc. µg/ml	Reten-tion time	Theroti-cal plate	Area	Mean area	SD	%SD
1.	Change in flow rate	0.9	20	4.834	7266	1872188	18722331	1133.6	0.06054822
		0.7	20	5.635	7828	1873385			
		1.1	20	4.370	6708	1871119			
2.	Change in pH	3	20	4.834	7266	1872188	1872166	1993.59	0.10648564
		2.6	20	5.107	7119	1870162			
		3.4	20	4.967	7122	1874149			

F. Ruggedness

The ruggedness of analytical method is the measure of its capacity to remain unaffected by small but deliberate change in environmental factors (room temperature, humidity). The ruggedness results are within range (Table 6 and fig.4).

Table No. 6: Ruggedness of Bosentan Monohydrate.

Sr. no.	Concentration	Retention time	Asymmetry	Area
1	10	4.966	1.18	1021967
2	20	4.983	1.25	1870997
3	30	4.924	1.29	2653330
4	40	4.461	1.27	3431305
5	50	4.931	1.24	4189265

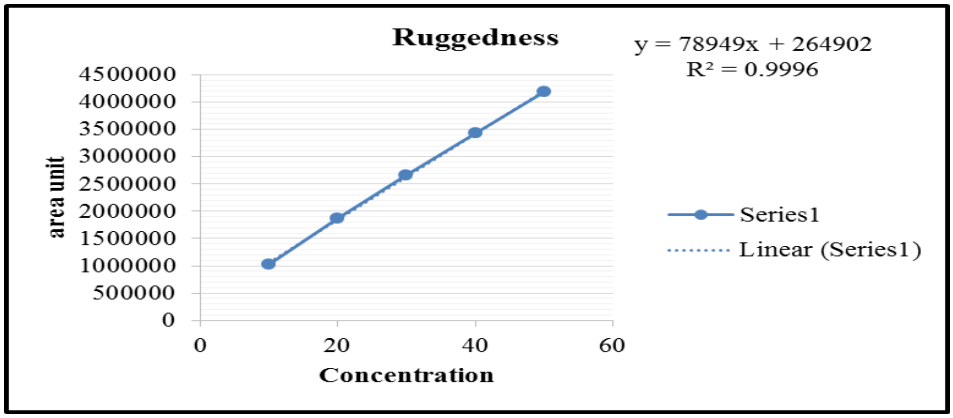


Fig. 4: Ruggedness plot of Bosentan Monohydrate.

G. Analysis of Marketed Tablet Formulation (Assay)

The proposed method was applied for the determination of Bosentan Monohydrate in marketed formulations (tablets) (Fig. 5) and the % recovery is found to be 99.8774% (Table 7). Accepted range is 98-102%.

Table No. 7: Assay Result of Bosentan monohydrate.

Drug	Label claim (mg/tab)	Concentration taken	Area		Amount found (mg/tab)	% assay
			standard	sample		
Bosentan monohydrate	62.5mg	30 ppm	2650075	2646827	62.42mg	99.8774

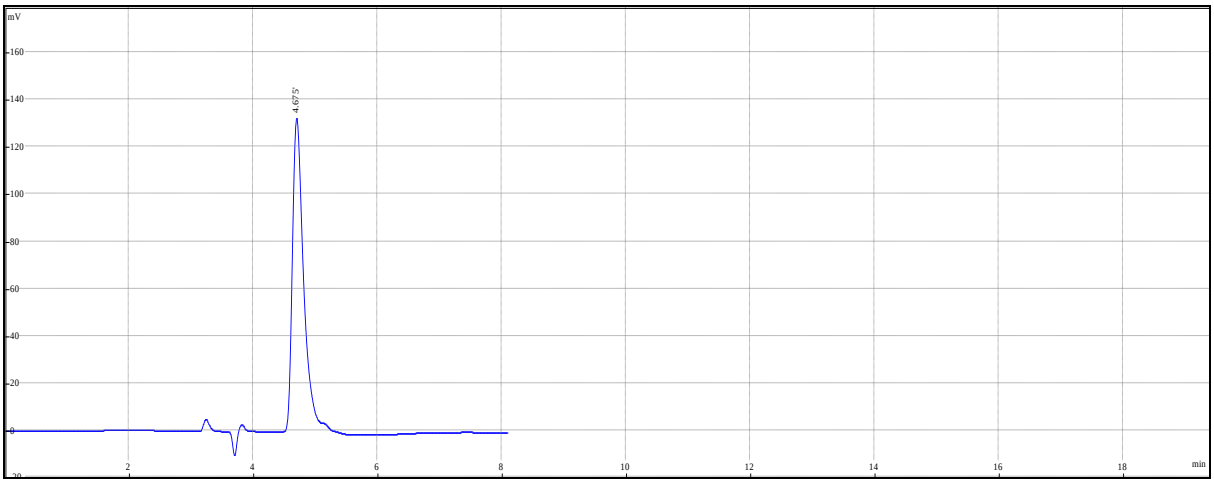


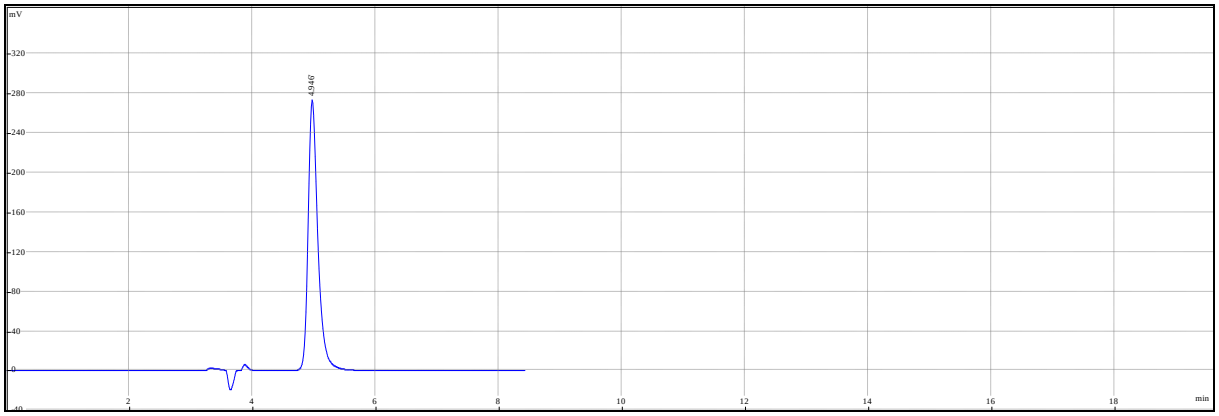
Fig. 5: Chromatogram of Assay.

H. Force Degradation Study

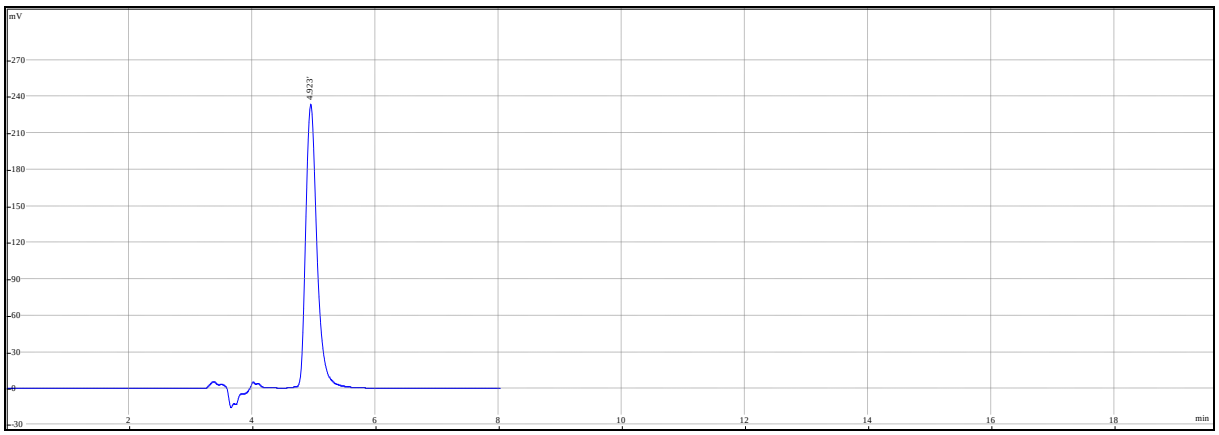
The stability indicating capability of the method was established from the separation of Bosentan Monohydrate peak from the degraded samples. Typical chromatograms obtained following the assay of stressed samples are shown in Figure 6A-6D. A very slight decomposition was seen on exposure of the drug solution to acidic, alkaline, oxidative and photolytic degradations indicating that the drug is very much resistant towards acidic, alkaline and oxidative reactions (Table 8).

Table No. 8: Results and Statistical Data of stress study.

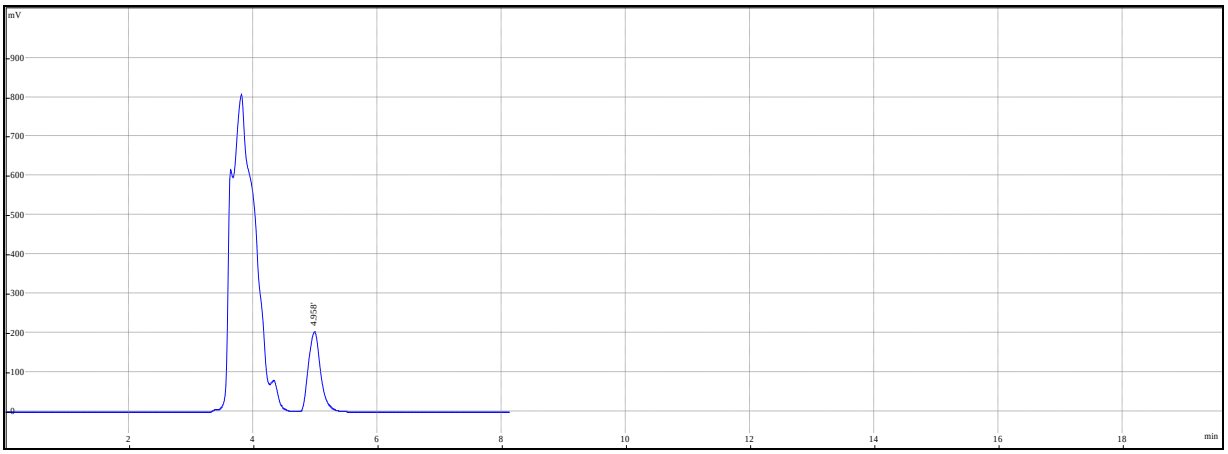
Sr. No.	Degradation	Area of Standard	Area of degraded Sample	Degraded up to %	Actual % degradation
1	**Acidic**	4186360	4017274	95.96702581	4.038974192
2	**Basic**	4186360	3908100	93.35317555	6.646824449
4	**peroxide**	4186360	3750216	89.58178465	10.41821535
5	**photolytic**	4186360	4174296	99.71182603	0.288173975



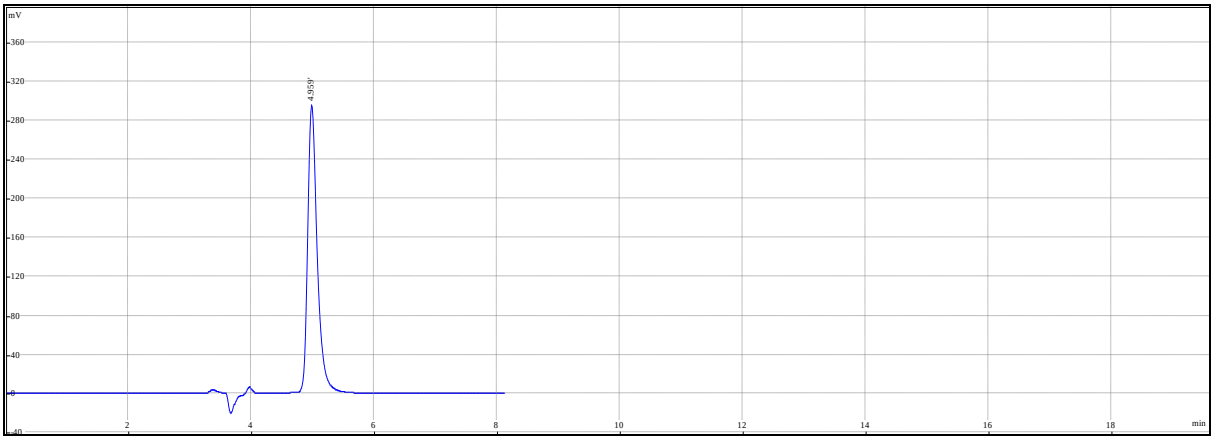
(A)



(B)



(C)



(D)

Fig. 6: Typical chromatograms of Bosentan Monohydrate (50µg/ml) (A) acidic degradation sample, (B) alkaline degradation sample, (C) oxidative degradation sample, (D) photolytic degradation sample.

The present stability-indicating method for the determination of Bosentan Monohydrate in pharmaceutical formulations is specific because the drug peak was well separated even in the presence of degradation products. Overall, the data demonstrated that the excipients and the degradation products did not interfere with the Bosentan Monohydrate peak, indicating the selectivity of the method. The proposed stability-indicating HPLC method was validated as per ICH guidelines. Bosentan Monohydrate is more resistant towards almost all types of degradations. During the oxidative degradation an extra peak was observed at 3.809mins along with the Bosentan Monohydrate peak. The chromatographic elution step was undertaken in a short time (< 5 min). The % RSD in precision, accuracy and robustness studies was found to be less than 2 indicating that the proposed method is precise, accurate and robust. The method was found to be specific as the drug peak elution did not interfere with any degradants during the forced degradation studies.

4 CONCLUSION

The present work involved the development of simple, accurate, precise, cost effective and suitable RP-HPLC method for estimation of drug in pharmaceutical dosage forms.

The method has several advantages, including simple mobile phase, low cost solvent, rapid analysis. The regression coefficient (r^2) for analyte is not less than 0.999 which shows good linearity. The % RSD in precision, accuracy and robustness studies was found to be less than 2 indicating that the proposed method is precise, accurate and robust.

As the drug peak was well separated even in the presence of degradation products the method is more specific. The percentage of degradation in all types of degradations is less and therefore it is concluded that Bosentan is more resistant towards acidic, alkaline, oxidative, thermal and photolytic degradations. The proposed method was validated as per ICH guidelines and can be successfully applied to perform long-term and accelerated stability studies of Bosentan even in pharmacokinetic studies. Since the method does not require use of expensive reagent and also less time consuming, it can be performed routinely in industry for routine analysis of marketed product of Bosentan Monohydrate tablet.

5 ACKNOWLEDGEMENT

I am grateful to all people who have helped and inspired me during my M. Pharmacy research work. It is a pleasure to convey my gratitude to them all in my humble acknowledgment. I am very much thankful to the principal, MVP Samaj's College of pharmacy, Nasik, for providing infrastructure and all the necessary facilities for this research work.

Once again I would like to thank all the people mentioned & those that I have unknowingly failed to mention but have been instrumental in the culmination of this project into a successful endeavor.

REFERENCES

1. Lavudu P, Avula PR, Are PC, Sekaran CB. Determination of Bosentan in Pharmaceutical dosage forms by high performance liquid chromatography. International Journal of Drug Delivery, 2013; 5: 146-151.
2. Rizwan SH, Sastry VG, Imad Q. Stability Indicating Method Development and Validation of Bosentan in Bulk Drug and Formulation by RP-HPLC Method. International Journal of Pharm Tech Research, 2015; 8(4): 569-579.
3. Jagtap NS, Vanjari SS and Bhosale SD. Stability-indicating Spectrophotometric method of Bosentan in Bulk and Pharmaceutical dosage form. World Journal of Pharmacy and Pharmaceutical Sciences, 2016; 5(7): 1420-1427.
4. ICH, Q2 (R1); Validation of Analytical Procedure: text and methodology. International Conference on Harmonization, Geneva, 2005; 1-13.

5. ICH, Q1A (R2), International Conference on Harmonization: Stability Testing of New Drug substance and Product, Geneva, 2003; 1-16.
6. ICH, Q1 B Stability Testing: Photostability Testing of New Drug Substance and Product, International Conference on Harmonization, Geneva, 2003; 1-8.
7. Annapurna MM, Pavani S, Anusha S and Mahanti H. Validated Stability Indicating RP-HPLC Method for the determination of Bosentan in presence of degradation products. Indo American Journal of Pharmaceutical Research, 2013; 3(6): 4528-4540.
8. Skoog DA, Holler FJ and Nieman TA. Principle of Instrumental Analysis, 5th Ed., Thomson Publication Limited, London, 2004; 103.
9. Chatwal G.R. and Anand S.K. Instrumental Method of Chemical Analysis, 5th Ed., Himalaya Publishing House, Mumbai, 2005; 25.
10. Willard HH, Merrist LL, Dean AJ, Frank A and Settle AF. Instrumental Methods of Analysis, 7th Ed., New Dehli; CBS Publishers and Distributors, 1986; 2-3, 585-587.
11. Beckett AH and Stenlake JB. Practical Pharmaceutical Chemistry, Part-II, 4th Ed., New Dehli; CBS Publishers and Distributors, 2004; 85-174.
12. Munson JW. Pharmaceutical Analysis, Modern Methods (Part B), Marcel Dekker, New York, 2000; 15-70.
13. Sethi PD., High Performance Liquid Chromatography, Quantitative Analysis of Pharmaceutical Formulations, 1st edition, New Delhi; CBS Publishers and Distributors, 2001; 3-11, 116-120.
14. Kasture AV, Wadodkar SG, More HN and Mahadik KR. Pharmaceutical Analysis, Instrumental Methods, Vol. II, Nirali Publication, 2012; 1.1.
15. Snyder LR, Kirkland JJ and Glajch JL. Practical HPLC Method Development, 2nd Ed., England; A Wiley-Interscience Publication, John Wiley and Sons, 1997; 02-05.
16. The United State Pharmacopoeia NF; US Pharmacopoeial convention Inc., Rockville, 2007; 2438.
17. Venkataraman S. Manasa M. Article Forced degradation studies: Regulatory guidance, characterization of drugs, and their degradation products - a review. Drug Invention Today, 2018; 10(2): 137-146.
18. Rao BV, Sowjanya1 GN, ajitha A, Rao VU. A review on stability indicating HPLC method development. World journal of pharmacy and pharmaceutical sciences, 2015; 4(8): 405-423.
19. Rawat T, Pandey IP. Forced degradation studies for Drug Substances and Drug Products- Scientific and Regulatory Considerations. Journal of Pharmaceutical Science and Research, 2015; 7(5): 238-24.
20. Blessy Mn, Patel RD, Prajapati PN, Agrawal YK, srivasta RK. Development of forced degradation and stability indicating studies of drugs—A review. Journal of Pharmaceutical Analysis, 2014; 4(3): 159–165.
21. Srivastava RK, Kumar SS, An updated review: Analytical Method Validation. European Journal of Pharmaceutical and Medical Research, 2017; 4(9): 774-784.
22. Petikam L, Avula PR, Sekaran CB, Ramesh A and Kumar AA. Application of Spectrophotometry and High Performance Liquid Chromatography for the Analysis of Bosentan in Pharmaceutical Dosage Form Chemical Science Transactions, 2014; 3(4): 1242-1253.
23. Siddappa k, and Hanamshetty PC. Development and Validation of High Performance Liquid Chromatography (HPLC) method for the Determination of Bosentan in Pure and Formulated Forms, Scholars Research Library Der Pharmacia Lettre, 2016; 8(8): 404-411.
24. Indian pharmacopoeia; vol.I; Govt. of India, Ministry of Health and Family Welfare. New Delhi; Pubished by Indian Pharmacopoeia Commission, Ghaziabad, 2010; 604.
25. Bhardwaj SK, Dwivedia K, Agarwala D.D. A Review: HPLC Method Development and Validation. International Journal of Analytical and Bioanalytical Chemistry. Universal Research Publications, 2015; 5(4): 76-81.
26. Parajuli RR*, Dahal P, Pokhrel P, Acharya1 CP, Koirala BR. force degradation study and its importance in formulation development- a review. Advanced Journal of Pharmaceutical and Life science Research, 2016; 4(2): 43-53.
27. Shahul HM, Jat RK, Indulekha VN. Method Development and its Validation for quantitative determination of Bosentan in tablet dosage form by RP-HPLC. Journal of Drug Delivery & Therapeutics, 2017; 7(2): 85-95.
28. Toomula N, Kumar A, Kumar S, Bheemidi VS. Development and Validation of Analytical Methods for Pharmaceuticals & Bioanalytical Techniques, 2011; 2(5): 1-4.
29. Santhosh G, nagasowajanya G, Ajitha A, Rao YU. HPLC Method Development and Validation: an overview. International Journal of Pharmaceutical Research & Analysis, 2014; 4(4): 274-280.
30. Selvadurai M, Kumar J. Simple Estimation of Bosentan in Tablet Formulation by RP-HPLC. American Journal of Analytical Chemistry, 2012; 3: 715-718.
31. Khan MA, Sinha S, Todkar M, Parashar V, Reddy KS. Development and Validation of a Stability Indicating analytical method for the related substances of Bosentan drug substance by HPLC. American Journal of Scientific and Industrial Research, 2012; 3(2): 69-80.
32. Lakshmi SRP. A Review on Chromatography with High Performance Liquid Chromatography (HPLC) and its Functions. Research and Reviews: Journal of Pharmaceutical Analysis, March 2015; 4(1): 1-15.

33. Panda A, Kulkarni S, Tiwari R. Stability studies: an integral part of drug development process. International Journal of Pharmaceutical Research and Bio-science, 2013; 2(6): 69-80.
34. Arunachalam A, Shankar M. stability studies: a review A. Asian Journal of Pharmaceutical Analysis and Medicinal Chemistry, 2013; 1(4): 184-195.
35. Gupta V, Jain AD, Gill NS, Gupta K. Development and validation of HPLC method - A Review. International Research Journal of Pharmaceutical and Applied Sciences, 2012; 2(4): 17-25.