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Development and Validation of RP-HPLC Method for the Dissolution Study of Bosentan in Bulk and in Pharmaceutical Dosage Form

Karunakaran Anandakumar[*1], Munusamy Jambulingam[1], Jayaprakash Rmaesh[1], Sebasthiar Josephine Subarla[1], Premkumar Sangeetha[1]and Murugesan Raja[2]

[1]Department of Pharmaceutical Analysis, Swamy Vivekanandha College of Pharmacy, Namakkal (Dt) Tamil Nadu, India
[2]Department of Pharmaceutics, Sri Ramakrishna Institute of Paramedical Sciences, College of Pharmacy, Coimbatore-641 044, Tamil Nadu, India

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Abstract

A simple, sensitive, precise and accurate reversed phase high performance liquid chromatographic (RP-HPLC) method has been developed for the dissolution study of Bosentan in bulk and in pharmaceutical dosage forms. The method was developed using the mobile phase comprising of Triethylamine buffer pH adjusted to 2.5 with ortho phosphoric acid and acetonitrile in the ratio of 50:50 v/v over Waters Symmetry C_8 column (150 mm length x 4.6 mm i.d x 5 µm particle size) at temperature 40°C. The flow rate was 1.5 ml/ min and UV detection was at 266 nm. The retention time of Bosentan was 4.475 mins. The recovery was found to be 99.98 % which is demonstrative of accuracy of the protocol. Inter-day and intra-day precisions of the newly developed method were less than the maximum allowable limit (%RSD < 2) according to ICH guidelines. The method showed linearity in the concentration range of 35-210 µg/ ml with correlation coefficient (r^2) value of 0.9999. No interference was observed from the blank (dissolution medium) and placebo samples. Hence the method was specific for determination of % release of Bosentan tablets in dissolution study. In robustness study for dissolution condition there was no significant change was observed in % release after individually changing the dissolution parameters. Calculations for system suitability parameters met the acceptance criteria. The percentage drug release was found to be 99.41 % for marketed Bosentan tablet. Several trials were performed by changing the dissolution medium; the 1 % SLS dissolution medium shows successful drug release. All the validation parameters were within the acceptance range. Therefore, the method was found to be simple, precise, accurate, reproducible, sensitive and less time consuming and can be successfully applied for routine quality control and analysis and dissolution study of Bosentan in bulk and in pharmaceutical dosage form.

Keywords: Bosentan, RP-HPLC, Method development, Validation, ICH guidelines, Dissolution studies.

[*]Corresponding author: Tel.: +91 9443454675 Fax: +914288-234417
E-mail: anandkarunakaran@gmail.com

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1. Introduction

Bosentan (BOS) is chemically, 4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-[2,2]-bipyrimidine-4-yl]-benzenesulfonamide (Figure 1). BOS is used to treat pulmonary artery hypertension [1] and to reduce number of digital ulcers [2]. BOS is an Endothelin receptor antagonist, which belongs to a class of highly substituted pyrimidine derivatives [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. BOS is a specific and competitive antagonist at Endothelin receptor types ET_A and ET_B. BOS has a slightly higher affinity for ET_A receptors than for ET_B receptors. Inhibition of the ET receptors disrupts the intracellular pathway that leads to vasoconstriction thereby causing vasodilation. BOS is white to yellowish white powder, freely soluble in dimethylformamide, acetonitrile; soluble in ethanol and ethyl acetate, slightly soluble in isopropanol, and very slightly soluble in hexane, poorly soluble in water [4-8].

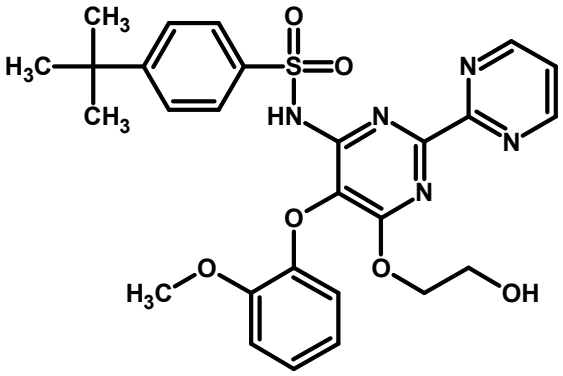


Figure 1. Chemical Structure of Bosentan

Literature survey revealed that few analytical methods have been reported for the estimation of BOS by UV-Visible spectroscopic methods [9-12] and RP-HPLC methods [13-18] Karnaker reddy *et al*. [13] reported RP-HPLC method development and validation of BOS drug present in tablets using methanol : potassium dihydrogen orthophosphate buffer pH 7.8 (60:40 % v/v) as mobile phase, at a flow rate of 0.8 ml/ min at 220 nm, Rt 3.702 mins. Muralidharan *et al*. [14] reported a simple RP-HPLC method for the estimation of BOS in tablet formulation using Acetonitrile: 10 mM ammonium acetate buffer pH 4.5 (70:30 % v/v) as mobile phase at a flow rate of 1 ml/ min at 265 nm, Rt 3.702 mins. Sujatha *et al*. [15] reported a new validated RP-HPLC method for the estimation BOS in tablet dosage form using phosphate buffer pH 4.0 and Acetonitrile (30: 70 % v/v) as mobile phase at a flow rate of 1 ml/ min at 270 nm, Rt 3.54 mins. Lavudu *et al*. [16] reported a determination of Bosentan in pharmaceutical dosage forms by HPLC using ammonium pH 5.0 : Acetonitrile (70:30 % v/v) as mobile phase at a flow rate of 1 ml/ min at 220 nm, Rt 1.986 mins. Kalaichelvi and Jayachandran [17] reported RP-HPLC method for Bosentan using phosphate buffer pH 5 : Acetonitrile (45:55 % v/v) as mobile phase at a flow rate of 1 ml/ min at 270 nm, Rt 5.7 mins. Lavudu *et al*. [18] reported an application of spectrophotometry and HPLC for Bosentan using methanol and ammonium buffer (60:40 % v/v)

as mobile phase at a flow rate of 1.0 ml/ min, λmax 227 nm, Rt 2.449 mins. Stability indicating HPLC [19-21] and HPTLC [22] methods were reported for the estimation of BOS. Also impurity profiling by RP-HPLC [23], determination of residual solvents by headspace gas chromatography [24, 25], determination of BOS in rat plasma by UFLC-MS/MS [26] method were reported. Literature survey revealed that there was no such simple RP-HPLC method for the dissolution study of BOS in bulk and pharmaceutical dosage form. Therefore, development of a simple RP-HPLC method for dissolution study of BOS is highly desirable. Hence, the aim of the present study was to develop a simple and rapid RP-HPLC method for dissolution study of BOS in bulk and in pharmaceutical dosage form and to validate the method according to ICH guidelines [27].

2. Materials and Methods

2.1 Chemicals and reagents

Working standard of BOS USP was obtained from Par Formulation Pvt Ltd. The marketed formulation was obtained from the local market. HPLC grade acetonitrile and methanol was procured from Fisher scientific (Hyderabad, India) and ortho phosphoric acid, triethylamine, sodium lauryl sulfate, sodium dihydrogen phosphate, sodium hydroxide of AR grade were purchased from Rankem (Hyderabad, India) and Merck (Mumbai, India). High purity water was obtained by using Millipore Milli-Q water purification system (Billerica, USA). The buffer was prepared by dissolving 1 ml of triethylamine in 1,000 ml of Milli-Q water and the pH was adjusted to 2.5 with orthophosphoric acid. One percentage of SLS was prepared by dissolving 10 gm of sodium lauryl sulfate in 1000 ml of Milli-Q water.

2.2 Instrumentation and chromatographic conditions

High Performance Liquid Chromatographic system (Waters Alliance, USA), equipped with an auto sampler and PDA detector was used for the analysis. The data was recording using Empower 3 software. Dissolution apparatus (Distek, USA) was used for the dissolution study of BOS. Analytical reversed phase C_8 (Waters Symmetry column, 150 mm length x 4.6 mm i.d x 5 µm particle size) was used for the separation. Mobile phase consisting of a mixture of triethylamine buffer adjusted pH to 2.5 with orthophosphoric acid and acetonitrile 50:50 v/ v was delivered at a flow rate of 1.5 ml/ min with PDA detection at 266 nm. The mobile phase was filtered through 0.45 µm membrane filter, sonicated and degassed before use. The column temperature was 40 ˚C.

2.3 Method development and optimisation of chromatographic conditions

Mobile phase comprising of different solvent composition were tried to achieve optimum separation. The mobile phase consisting of Triethylamine buffer, pH-adjusted to 2.5: Acetonitrile in the ratio of 80:20, 70:30, 60:40 and 50:50 % v/ v were tried. In the ratio of 80:20 % v/v and 70:20 % v/v the Bosentan peak was not eluted properly. At 60:40 % v/v the peak shape was good but slightly tailing was observed. At 50:50% v/v Bosentan was eluted with sharp peak and obeyed system suitability parameters. Finally, triethylamine buffer pH was adjusted to 2.5 with ortho phosphoric acid and acetonitrile (50:50 v/ v) was selected as an appropriate developing medium which gave good separation and met acceptable system suitability parameters.

2.4 Selection of column oven temperature:

The Study was started with initial stage column temperature maintained at ambient, and then increased slowly up to 40 °C. At 40°C good peak shape, peak symmetry and better separation was observed. The peak shape and peak symmetry was good at 40 °C than the ambient temperature. Hence, 40 °C temperature was selected for further analysis.

2.5 Preparation of standard solution
Standard stock solution of BOS was prepared by dissolving 35 mg of BOS in 25 ml of methanol in 25 ml volumetric flask. The solution was sonicated for 5 min. Five ml of the above stock solution was diluted to 50 ml with 1 % SLS to get a concentration range of 140 µg/ ml.

2.6 Preparation of sample solution
BOS tablet powder equivalent to 35 mg was accurately weighed and transferred into 25 ml volumetric flask, dissolved and make up the volume with methanol. The resulting solution was sonicated for 15 minutes and filtered through Whatman filter paper No. 41 to obtain the concentration of 1400 µg/ ml. Five ml of the above solution was transferred into 50 ml volumetric flask, dissolved and made up the volume with 1 % SLS to get a final concentration of 140 µg/ ml.

2.7 Dissolution study
Dissolution test for BOS formulation was performed in the dissolution system (n = 6), dissolution apparatus No II (paddle). The media used was 1 % SLS. The temperature of the bath was maintained at 37^0C and speed of the paddle was set at 50 rpm. The aliquots of sample were withdrawn up to 60 min in different time intervals, and filtered through 0.45 µ PVDF membrane filtered and injected into HPLC system.

3. Results and Discussion

All of the analytical parameters for the proposed method were determined according to the International Conference on Harmonization (ICH) guidelines.

3.1 Selection of wavelength
10 µg/ ml of BOS standard solution was scanned between 200-400 nm. The λmax was detected at 266 nm and this wavelength was fixed for analysis.

3.2 Optimization of chromatographic conditions
Mobile phase comprising of different solvent composition were tried to achieve optimum separation. The mobile phase consisting of Triethylamine buffer pH adjusted to 2.5: Acetonitrile in the ratio of 80:20, 70:30, 60:40 and 50:50 % v/ v were tried. In the ratio of 80:20 % v/v and 70:20 % v/v the Bosentan peak was not eluted properly. At 60:40 % v/v the peak shape was good but slight tailing was observed. At 50:50 % v/v Bosentan was eluted with sharp peak and obeyed system suitability parameters. Finally, triethylamine buffer pH adjusted to 2.5 with orthophosphoric acid and acetonitrile (50:50 v/v) was selected as an appropriate developing medium which gave good separation and met acceptable system suitability parameters.

3.3 Linearity
Linearity of the method was confirmed by constructing calibration graph. Seven solutions having concentrations of 35, 70, 112, 140, 168, 196 and 210 µg/ ml were prepared. Then 20 µl from each solution was injected using auto sampler and the analyses were monitored at 266 nm. The procedure was repeated for six times. The method was found to be linear in the concentration range of 35-210 µg/ ml with the correlation coefficient of 0.9999 which were found to be within the accepted range of guidelines and represented a good linear relationship of the newly developed method. The slope and intercept of the calibration curve was found to be 21908.463 and 4229.138, respectively. The LOD and LOQ were found to be 207.5537 µg/ ml and 628.9505 µg/ml, respectively. The calibration curve is shown in Figure 2.

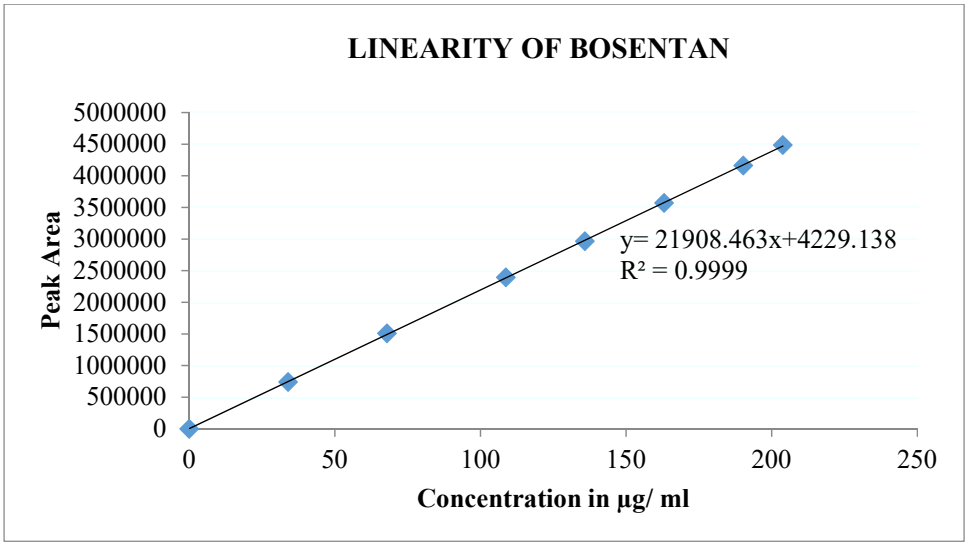


Figure 2. Linearity plot of Bosentan

3.4 Precision3

3.4.1 Repeatability

Repeatability of the method was done by using the different analysis of the dissolution sample for six times. The results are shown in Table 1.

Table 1. Repeatability studies

Sample No	Mean Peak Area*	% Release*	Average % Release	% RSD
1	2995113	98.3	98.93	0.43
2	3017075	99.0		
3	3023718	99.2		
4	3016147	99.0		
5	3032621	99.5		
6	3005952	93.6		

Remark: *Mean of six observations

3.4.2 Intermediate precision

Intermediate precision was done by intraday and inter day analysis of dissolution sample was done for three times in the same day and one time for three consecutive days. This is shown in Table 2.

Table 2. Intermediate precision

Parameters	% Release*	SD	% RSD
Intraday	99.69	0.1969	0.19
	99.74	0.2014	0.20
	99.97	0.1739	0.17
Inter day	99.32	0.1234	0.15
	99.62	0.1523	0.13
	99.53	0.1230	0.15

Remark: *Mean of six observations.

3.4.3 Reproducibility:

Reproducibility of the method was done by using the different analysts and different instruments. The dissolution sample was analysed with different analysts and the results are shown in Table 3.

Table 3. Reproducibility studies

Sample No	Analyst 1		Analyst 2	
	Peak area	% Release	Peak area	% Release
1	2995113	98.3	3215897	100.1
2	3017075	99.0	3186967	99.2
3	3023718	99.2	3149781	98.0
4	3016147	99.0	3205632	99.8
5	3032621	99.5	3186839	99.2
6	3005952	98.6	3204993	99.7
Mean		98.9		99.3
%RSD		0.43		0.74

Remark: *Mean of six observations

3.5 Detection Limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Based on the Standard Deviation of the Response and the Slope:

The detection limit (DL) was expressed as equation (1):

$$DL = \frac{3.3\ \sigma}{S}$$

Where, σ = the standard deviation of the response
S = the slope of the calibration curve
The slope S may be estimated from the calibration curve of the analyte.

3.6 Quantitation Limit:

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

Based on the Standard Deviation of the Response and the Slope:
The quantitation limit (QL) was expressed as equation (2):

$$QL = \frac{10\,\sigma}{S}$$

Where, σ = the standard deviation of the response
S = the slope of the calibration curve
The slope S may be estimated from the calibration curve of the analyte.

3.7 Accuracy

Accuracy of the method was confirmed by recovery studies. The accuracy was expressed by calculating the percent recovery of the analyte from dissolution sample and % RSD was calculated. To evaluate the accuracy of the proposed method, successive analysis of four different concentrations of 25 %, 50 %, 100 % and 150 % standard BOS were carried out using proposed method. The recovery studies were performed in triplicate. The percentage recovery and % RSD were found to be 99.9638 % and 0.0516 %, respectively. This indicates that there was no interference due to excipients used in formulation. Hence, the proposed method was found to be accurate (Table 4).

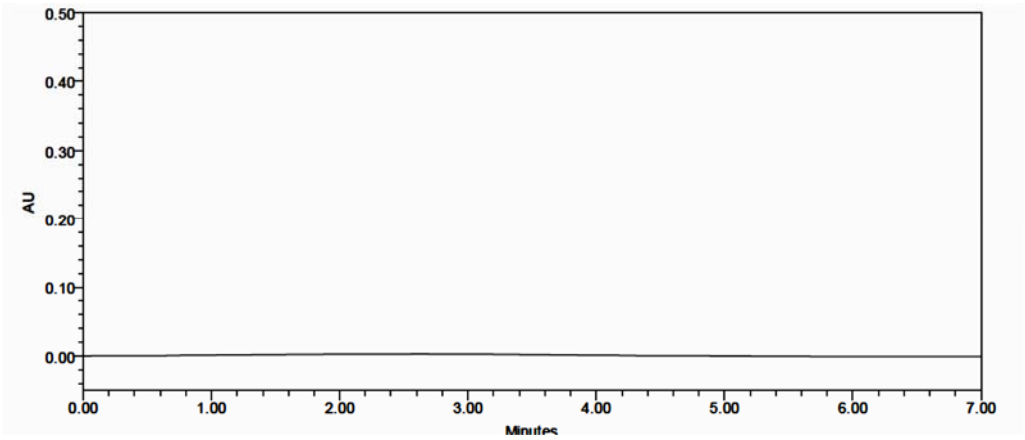
Table 4. Accuracy of the method for the determination of Bosentan

Spiked level	Amount Added * (mg)	Amount Recovered* (mg)	Peak Area*	% Recovery	Mean (%) Recovery	SD	% RSD
25 %	30.042	29.993	730938	99.83	99.96	0.05	0.05
25 %	30.119	30.119	734019	100.00			
25 %	30.139	30.139	734518	100.00			
50 %	60.307	60.300	1469540	99.99			
50 %	60.220	60.187	1466788	99.95			
50 %	60.278	60.237	1467998	99.93			
100 %	120.595	120.590	2938812	100.00			
100 %	120.846	120.843	2944988	100.00			
100 %	120.759	120.661	2940543	99.92			
150 %	181.173	181.165	4415051	100.00			
150 %	181.057	181.045	4412127	100.00			
150 %	181.134	181.088	4413178	99.97			

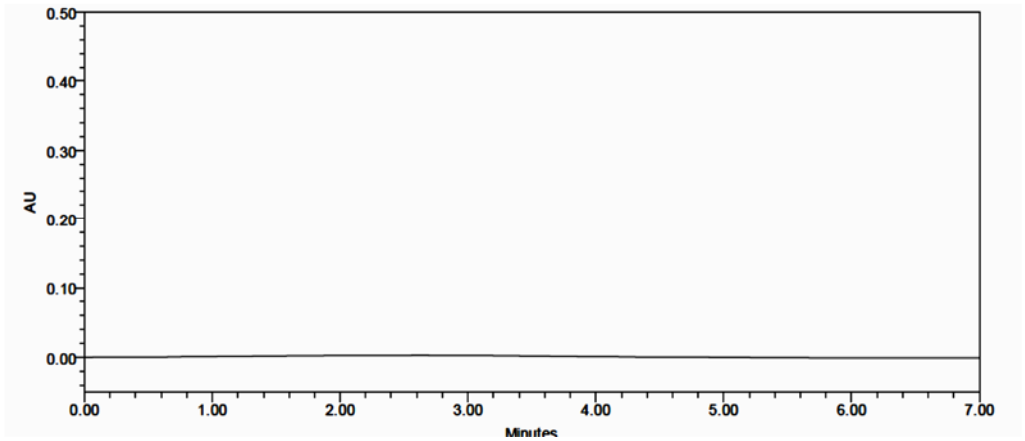
Remark: *Mean of six observations.

3.8 Specificity

For the specificity study, the blank, placebo, standard and sample solution were prepared and injected for the determination of interference in the retention time of BOS peak. No interference from blank and placebo was observed at the retention time of the BOS. Therefore, it was concluded that the method was specific and can assess unequivocally the analyte of the interest in the presence of possible interference. The chromatograms for the blank, placebo, standard and sample are shown in Figure 3.

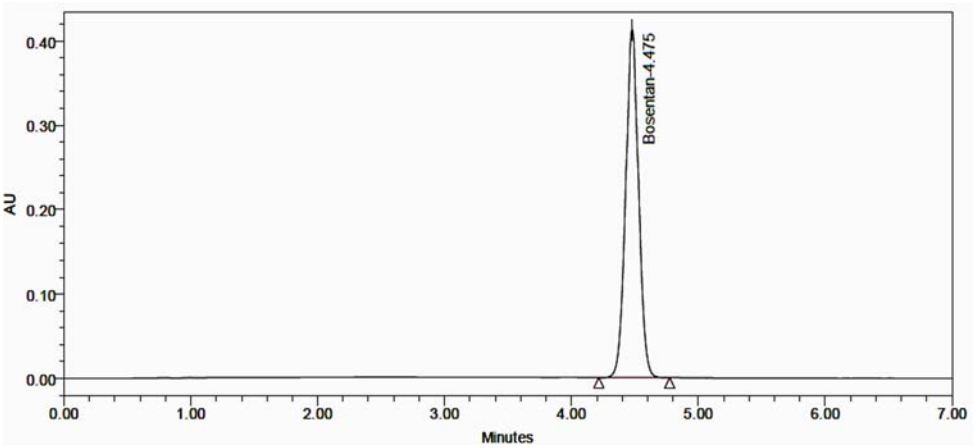


(a)

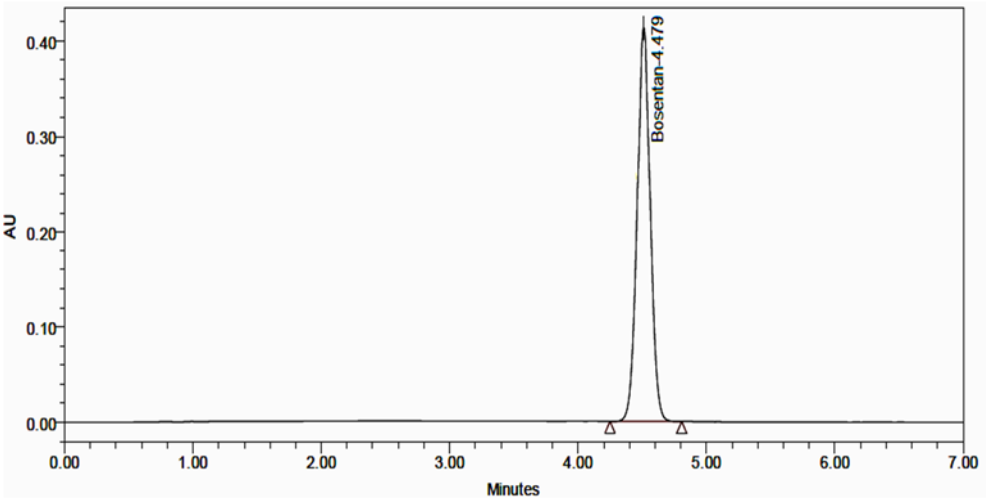


(b)

Figure 3. Chromatogram (a) blank, (b) placebo, (c) standard, (d) sample



(c)



(d)

Figure 3. Chromatogram (a) blank, (b) placebo, (c) standard, (d) sample (cont.)

3.9 System suitability test

System suitability test was used to verify the precision/ reproducibility of the system that is adequate for the analysis to be performed. Parameters such as theoretical plates, tailing factor and reproducibility (% RSD for peak area of five replicates) were determined and compared against specifications. Five replicate injections of standard BOS solution were run using HPLC. Theoretical plate and tailing factor were determined. The theoretical plates were found to be 7829 and tailing factor was found to be 0.97 which were found to be within the limit (Table 5).

Table 5. System suitability parameters

S. No	Parameter	Obtained value	Acceptable limit
1	Retention time (min)	4.475	-
2	Theoretical plates	7829	>2000
3	Tailing factor	0.97	< 2
4	%RSD	0.3115	< 2

3.10 Robustness

Robustness of the method was checked by making slight changes in chromatographic and dissolution conditions like mobile phase ratio, pH of buffer, flow rate, temperature, medium volume, medium strength, and rpm. It was observed that there were no marked changes in the chromatograms, which demonstrated that the developed RP-HPLC method was found to be robust (Table 6).

Table 6. Robustness

Parameter		Retention Time*	Peak area*	% RSD	USP Tailing
Flow Rate	1.35 ml/ min	5.156	3313367	0.06	0.91
	1.65 ml/ min	4.247	2708208	0.08	0.91
Column Temp	35^0C	4.770	2978422	0.10	0.92
	45^0C	4.541	2975434	0.13	0.90
Mobile phase	48:52	4.007	2968468	0.05	0.92
	52:48	5.476	2973257	0.15	0.90
Normal Buffer pH	2.3	4.677	2961037	0.06	0.91
	2.7	4.820	2957455	0.04	0.91
Wavelength	264	4.474	2987501	0.44	0.91
	268	4.480	3013954	0.43	0.91
Strength of Medium	0.8% SLS	4.476	2994092	0.97	0.91
	1.2% SLS	4.483	3013470	0.69	0.91
Volume of Medium	882 ml	4.473	3050004	1.16	0.91
	918 ml	4.478	2941830	1.49	0.91
RPM	48	4.471	3000895	0.56	0.91
	52	4.479	3041987	0.38	0.91

Remark: *Mean of six observations

3.11 Filter study

A filter study was performed to determine the suitability of filter used and to determine the amount of filtrate to be discarded before a sample solution was collected for analysis. The peak area found in the filtered fractions of sample solution was comparable to the peak area found in the centrifuged portion of sample solution. There was no significant difference in peak area between different volumes filtered. Therefore the 0.45 µm PVDF and Nylon filter were suitable for use and the discarding of 4 ml of sample solution as filtrate as stated in the method (Table 7).

Table 7. Filter Study

Sample Name	Peak Area	% Difference
Centrifuged (10 min @ 3500rpm)	3002048	NA
0.45µ PVDF filtered, 3 ml discarded	3008720	0.2200
0.45µ PVDF filtered, 4 ml discarded	3009031	0.2300
0.45µ PVDF filtered, 5 ml discarded	3013272	0.3700
0.45µ PVDF filtered, 6 ml discarded	3011554	0.3100
0.45µ Nylon filtered, 3 ml discarded	3002468	0.0100
0.45µ Nylon filtered, 4 ml discarded	3008625	0.2100
0.45µ Nylon filtered, 5 ml discarded	3015016	0.4300
0.45µ Nylon filtered, 6 ml discarded	3005939	0.1200

3.12 Dissolution

The method was applied to dissolution study of BOS. The percentage drug release was found to be 99.41 % for Bosentan tablet 125 mg and 99.99 % for Marketed Bosentan Tablet 125 mg. Several trials were performed by changing the dissolution medium, the 1% SLS dissolution medium showed successful drug release. The results are shown in Table 8.

Table 8. Determination of percentage release in marketed formulation

Injection	Label claim (mg)	% Drug Release
Tracleer 125 mg	125 mg	99.99 %
Bosentas	125 mg	99.98%
Lupibos	125 mg	98.64%

3.13 Solution stability

The stability of both standard and sample solutions was studied by observing the peak areas of both the drugs. The amount calculated at different time intervals was within the acceptable limit. The results indicate that the solutions were stable up to 48 hours. The stability data is illustrated in Table 9.

Table 9. Solution stability of sample at room temperature

Time	Peak Area*	Retention Time	% Release*	% Difference
Initial	2995113	4.481	98.3	NA
24 h	2990682	4.483	98.1	0.14
48 h	2983104	4.485	97.9	0.40

3.14 Comparison of the developed method with the reported methods

Lavudu *et al*. [16, 18] reported two methods for the estimation of BOS by RP-HPLC but the retention times were found to be 1.986 mins and 2.449 mins, respectively. When calculating capacity factor, it was low and the separation was not good. Hence, the developed method had more advantageous than the reported methods. Karnakar reddy *et al.* [13] selected the detection wavelength as 220 nm. But the λmax of BOS was more than 265 nm. Hence, the detection was not clearly done in the reported method. The developed method was validated accordance with ICH guidelines and met all the acceptance criteria. The system suitability parameters calculated also in the accepted range. Hence the developed method can be used for the effective routine quality control analysis and dissolution study of BOS in bulk, tablet dosage form and in dissolution medium.

4. Conclusions

A simple, precise, accurate and reproducible RP-HPLC method has been developed and validated for the quantitative determination of Bosentan in the dissolution sample. A complete dissolution of BOS could be achieved after 45 min using apparatus II (paddle) at 50 rpm in 900 ml of dissolution medium (1 % Sodium lauryl sulphate). Based on the above studies, it was concluded that the developed RP-HPLC method was specific, accurate, precise, rugged, robust and linear over the concentration range. The report obtained in the validation parameter met the respective acceptance criteria. The results were statistically validated. Hence, the developed method can be used for routine quality control analysis and dissolution study of Bosentan in bulk and in pharmaceutical dosage form.

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