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Development and Validation of High Performance Liquid Chromatography (HPLC) method for the Determination of Bosantan in Pure and Formulated Forms

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ABSTRACT

A reversed-phase high performance liquid chromatographic (RP-HPLC) method for the determination of Bosantan (BOS) in bulk material and pharmaceutical formulations has been developed and validated. The chromatographic separation of BOS was performed on an Inertsil C_8 column (5 μ particle size, 15 cm length x 4.6 mm i.d) with CLC ODS (4 cm x 4.6 mm, i.d.) as a guard column to protect analytical column, a mobile phase consisting of acetate buffer (pH 5.5) acetonitrile in the ratio of 20:80 (v/v) at a flow rate of 1.0 ml/min at ambient temperature using UV detector at 230 nm. The developed method gave good retention time of 2.225 min. The method was found to be specific to BOS and able to resolve the drug peak from formulation excipients. The calibration curve was noticed to be linear over the concentration range of 5-150 µg/ml (R^2 = 0.9999). The method was validated statistically for its selectivity, linearity, precision, accuracy and robustness. In view of this, the method could be employed as a more convenient and efficient option for the analysis of BOS in drug substance and formulations.

Key words: Bosantan, HPLC, Method development, Validation, Anti-hypertensive,

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INTRODUCTION

Bosentan (4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl]benzene-1-sulfonamide), a nonpeptidic dual endothelin receptor antagonist (ERA) has molecular formula of $C_{27}H_{29}N_5O_6$ S with relative molecular mass of 551.614 g mol^{-1}. It is the first orally active drug approved by United States Food and Drug Administrative as Tracleer (125 mg) for the successful treatment of pulmonary arterial hypertension (PAH).

Tracleer improves the exercise ability and decreases the rate of clinical worsening in patients with WHO Class III or IV symptoms of PAH, by blocking the binding of endothelin to its receptors, thereby negating endothelin's deleterious effects [1–8]. Further Tracleer has been demonstrated to be effective in remodelling the pulmonary vascular tree through several mechanisms including vasodilatation, antifibrotic and antithrombotic actions [9]. A comprehensive literature survey revealed that there are several bio analytical HPLC methods for the determination of bosentan monohydrate and its metabolite in blood plasma, whereas, there are few other literatures disclosed only for the quantitative determination of bosentan in biological and formulation samples [10–13]. The reported HPLC method [13] was not capable to separate the peaks of impurities and bosentan. The literature survey also revealed that there was no stability-indicating RP-LC method for the determination of process and degradation-related impurities formed under the stress conditions in bosentan monohydrate.

In this paper we describe development and validation of related substances method for accurate quantification of five potential process impurities in bosentan monohydrate samples as per International Conference on Harmonization (ICH) recommendations. Intensive stress studies are carried out on bosentan monohydrate; accordingly a stability indicating method is developed, which could separate various degradation products. The present active pharmaceutical ingredient (API) stability test guideline Q1A (R2) issued by ICH suggests that stress studies should be carried out on active pharmaceutical ingredient (API) to establish its inherent stability characteristics, leading to separation of degradation products and hence supporting the suitability of the proposed analytical procedures. It also recommends that the analytical test procedures for stability samples should be stability indicating and should be fully validated. Accordingly, the aim of present study is to establish degradation pathway of bosentan monohydrate through stress studies under a variety of ICH recommended test conditions. Development of an accurate and efficient analytical method for determining the quality and evaluating the impurity profile of drug substances is some of the critical activities carried out during process research and development in order to meet the requirements of various regulatory authorities [14, 15]. Hence, this paper provides a simple, rapid, selective, method for determining the bosentan monohydrate bulk drug and in its formulated form along with its validation as per USP and ICH guidelines [16, 17].

In the present work, we developed a simple and sensitive RP-HPLC method for quantitative determination of BOS in pure form and pharmaceutical formulations. The developed RP-HPLC method does not involve extraction of BOS from the excipients.

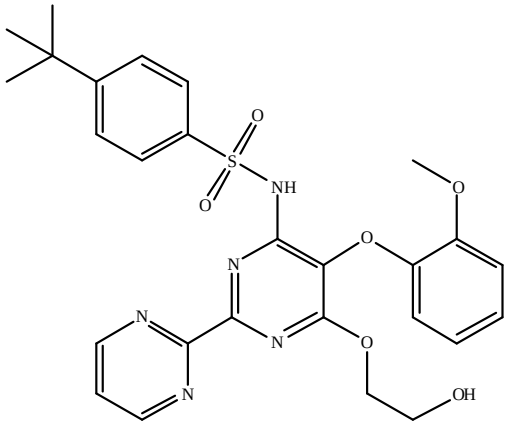

Figure 1: Structure of Bosantan

MATERIALS AND METHODS

1.1 Apparatus: Waters, binary pump with w2489 UV-detector.

1.1.1 Materials and reagents

HPLC grade acetonitrile and water were used. Analytical reagent grade sodium acetate and acetic acid were used. Tablets of BOS were procured from the local market.

1.1.2 Analytical procedures for solution preparations

a. Drug solutions

Bosentan pure (99.8%) obtained as gift sample from Cipla India Ltd. Mumbai, (10 mg of reference standard in 10 mL mobile phase (ACN:Buffer, 80:20) the apparent concentration is 1000 µg mL^{-1}) the lower concentrations of this solution are prepared by appropriate dilutions with mobile phase (100 µg mL^{-1}).

b. Standard working solutions

Working solutions of each 100 µg/ml of BOS was prepared separately in the mobile phase. Studies on the stability of analytes in standard working solutions showed that there were no decomposition products.

c. Preparation of buffer solution

Sodium acetate buffer (0.3 M) solution was prepared by dissolving 40.824 g of sodium acetate trihydrate (w/v) into a 1.0 liter volumetric flask and making up to volume with HPLC grade water. Then this solution of 273 ml was taken in a separate 1000 ml volumetric flask and added 28.5 ml of 1M acetic acid then made up to the mark with HPLC grade water, then the pH of the solution was adjusted to 5.5 using 1M acetic acid. This buffer solution was filtered through 0.22 µ Millipore filter.

d. Pharmaceutical preparations

Twenty tablets of BOS (Tracleer 125) were weighed separately and finely powdered. An accurately weighed quantity equivalent to 10 mg of the drug was transferred in to a 100 ml volumetric flask. Approximately 50 ml of the mobile phase was added and shaken for 10 min. and it was filtered first through Whatman No. 42 filter paper, then 0.45 µ Millipore membrane filter and the filtrate was made up to 100 ml with the mobile phase. Suitable amount of the aliquot was taken for assay studies. The method operating conditions were summarized in Table 1.

Table 1 : Method operating conditions

Column	:	Inertsil C_8 column (15 cm length x 4.6 mm i.d., 5 µ particle size,) with CLC ODS (4 cm x 4.6 mm, i.d.) as a guard column to protect analytical column
Mobile phase	:	Acetonitrile : Buffer (80:20, v/v)
Detector	:	UV
Wavelength	:	230 nm
Flow rate	:	1.0 ml/min
Temperature	:	$25^0C \pm 2^0C$ (ambient)
Mode	:	Reverse Phase
Membrane	:	0.22 µ Millipore
Run time	:	10 min

1.1.3 Recommended procedures

i. Chromatographic conditions

HPLC analysis was performed by isocratic elution with a flow rate of 1.0 ml/min. The mobile phase consisting of 0.3 M sodium acetate buffer-acetonitrile (20:80 v/v) [pH adjusted to 5.5 using 1M acetic acid] was used. All solvents were filtered through a 0.22 µ Millipore membranes filter before use and degassed in an ultrasonic bath. Volumes of 10 µl each of pharmaceutical samples were injected into the column. Effective quantification was achieved by measuring at 230 nm with UV detector. Chromatographic run time of 10 min was maintained throughout the study. The suitability of the chromatographic system was monitored by calculating the capacity factor (k^1), the resolution (R) was not needed because only one peak present, the selectivity (α) and the peak asymmetry (T). The chromatographic peak observed was of good shape and completely resolved from any other excipients from formulation. The chromatogram of pharmaceutical formulation samples did not show any co-eluting interference peak with the analyte.

ii. Establishment of calibration

Working standard solutions of BOS (5-150 µg/ml) containing fixed concentration was prepared in the mobile phase. Triplicate 10 µl injections were made for each standard solution to check the reproducibility of the detector response at each concentration level. The values of peak area ratio of standard to internal standard were plotted against the concentration of the drug to obtain the calibration graph. The results were subjected to regression analysis.

iii. Analysis of tablet

Appropriate amount of the drug solution obtained by following the procedure described for analysis of pharmaceutical preparations was taken and chromatogram was recorded. The chromatogram at 230 nm showed a complete resolution of the peak.

RESULTS AND DISCUSSION

i. Method development and Optimization of HPLC conditions

Reversed-phase HPLC method was chosen for the analysis of BOS. In this, the retention of analyte is related to its hydrophobicity. The more hydrophobic analyte, the longer it is retained on the column. When an analyte is ionized, it becomes less hydrophobic and therefore, its retention decreases. An acid loses a proton and becomes ionized when pH increases while a base gains a proton and becomes ionized when pH decreases. Therefore, when a separation

mixture containing acids and /or bases is subjected to reversed-phase HPLC, it is necessary to control the pH of the mobile phase using an appropriate buffer in order to achieve reproducible results. In the present study, acetonitrile was used as an organic modifier and 0.3 M sodium acetate as buffer. The separation and resolution were found to be pH dependent. In such cases the proper range of pH is generally selected by considering the pKa of the analyte.

ii. Mobile phase

Mobile phases for reversed-phase chromatography usually consist of an aqueous component, with or without a buffer in combination with an organic modifier such as methanol, acetonitrile, isopropyl alcohol, trietylamine and water. As BOS is lipophilic, the use of an organic modifier would affect its retention time and thus the effect of varying proportions of organic modifier, in the mobile phase, on retention times were investigated. The organic modifier used in these studies was acetonitrile and mobile phase compositions of 50, 65, 70 and 80 % (v/v) acetonitrile were evaluated. An increase in acetonitrile concentration resulted in a decrease in the retention time and a very sharp peak was obtained. As expected, the mobile phase compositions of 65 and 70 % (v/v) acetonitrile revealed longer retention times than those containing 80% (v/v). In addition, peak resolution was poor; therefore the optimum mobile phase 0.3 M sodium acetate (pH 3.5) acetonitrile (20:80 v/v) was selected. Under these experimental conditions sharp peaks were obtained for BOS at the retention time (t_R) of 2.225 which is much better retention times we have got for the typical fast HPLC analysis. Before starting the analysis we have run the method to equilibrate the column and also injected several blanks. The blank chromatogram shows less baseline drift as well as there was no minute peak at the bosantan retention time as shown in Fig. 2. A typical chromatogram obtained for pure drug samples is shown in Fig. 3.

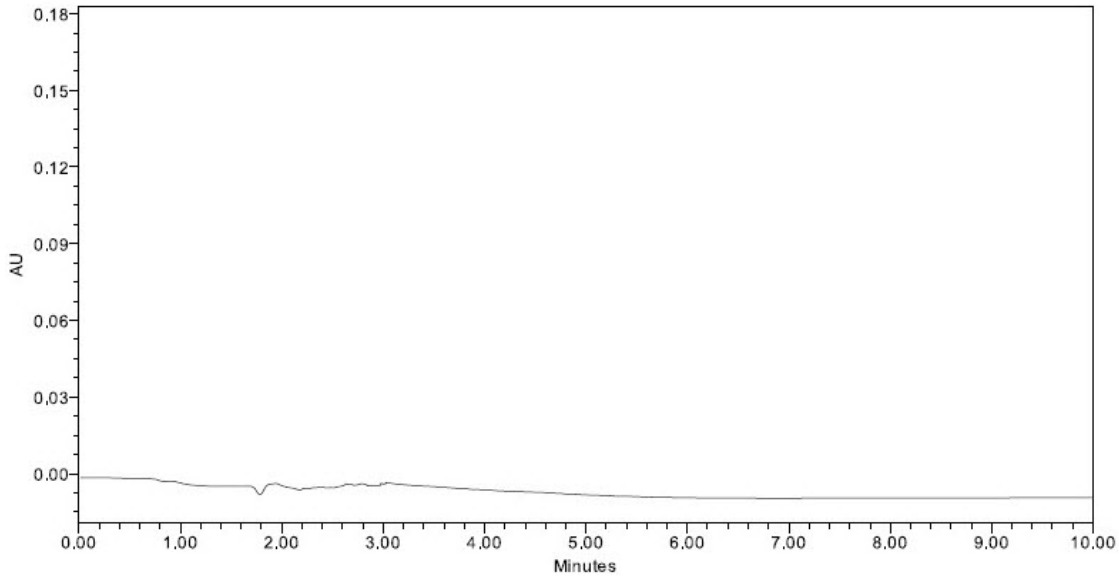

Figure 2: Blank solution chromatogram

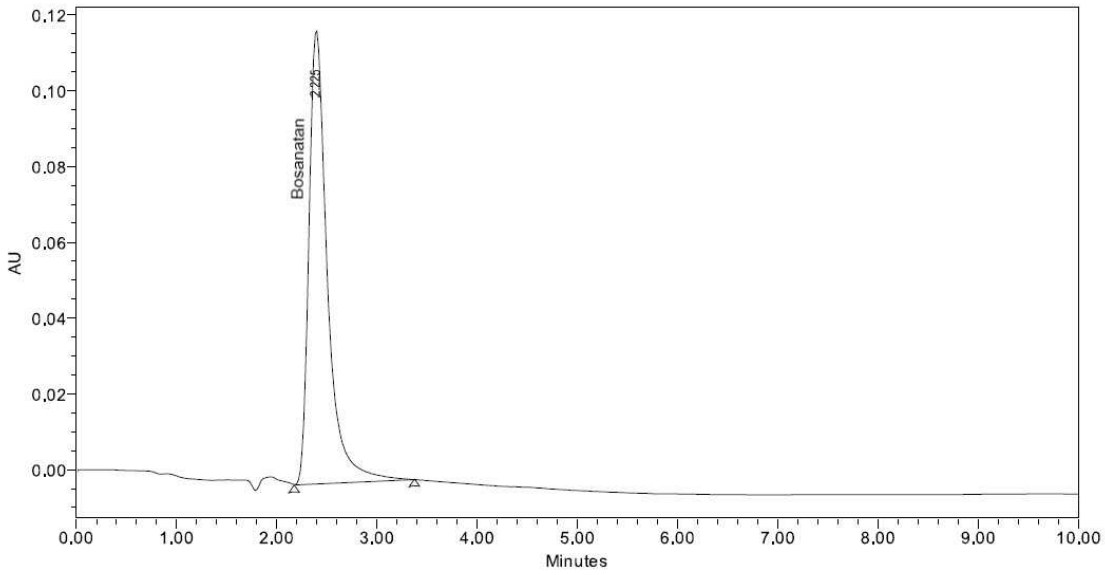

Figure 3: Bosantan reference chromatogram (100 ppm)

iii. Column

Polar compounds which have a high solubility in polar solvents are better separated in a reverse phase partition mode. BOS showed good resolution when chromatographed on a non-polar stationary phase used in conjunction with a polar mobile phase. Inertsil C8 (15 cm length x 4.6 mm i.d., 5 µ particle size), a reverse phase column was widely used for separation of a large number of organic compounds. The C8 columns are faster than the C18 because of less carbon load and it has been found that there was difference in the peak shape also. It was observed that this column gave satisfactory separation of BOS in the solvent system comprising of sodium acetate buffer and acetonitrile.

iv. Buffer pH

The effect of buffer pH was investigated empirically over the pH range of 3-7 and more specifically at pH values of 3, 4, 5, 6 and 7. The retention time (t_R) of BOS was affected by buffer pH due to its base nature. Due to the basic nature, these compounds tend to become less ionized as pH increase. Therefore they become less polar, thus exhibiting an increased preference for bonding to the stationary phase. In addition, as the pH of the buffer increased from pH 4 to pH 7, the resolution between BOS and the formulation excipients peaks, standard was compromised and considerable overlapping of peaks was observed, making quantitation difficult. In contrast, when a buffer of pH 5.5 was used, all peaks were well-resolved, sharp and symmetrical. It has been reported that the compounds with a pKa > 8 produce better peak shapes at pH 5.0 and thus this buffer of pH was selected for further use in these studies.

v. Flow rate

Flow rate of the mobile phase plays an important role in chromatographic determination of pharmaceutical drugs. The effects of flow rates in the range of 0.5 to 1.5 ml/min were examined in the determination of BOS. With increase in flow rate, the analyte was eluted quickly and the peak was not resolved properly. When flow rate was decreased, the peaks obtained were broad and required more time for elution. However, with a flow rate of 1.0 ml/min, an optimal signal to noise ratio with a reasonable separation time was noticed. Hence, a flow rate of 1.0 ml/min was maintained for ideal chromatographic conditions.

vi. Retention times

The reproducibility of the retention times of BOS was calculated based on the average of nine determinations. The retention times of BOS was observed to be 2.225 min. Thus, the total time of analysis was observed to be less than 10 min. This facilitated the analysis of BOS in short time.

vii.Wavelength

The solution containing BOS exhibited maximum absorption at 230 nm and hence, this wavelength was chosen for analysis.

viii. Suitability of the method

The chromatographic parameters such as resolution, selectivity and peak asymmetry were evaluated. The calculated resolution values R was not considered because of only one peak present and there was no other peak appears in the chromatogram. While the selectivity value (α) was observed to be 2.5626. The capacity factors (k^1) were found to be 0.8541. The peak asymmetry (T) values were noticed to be 1.05, the corresponding chromatographic parameters given in Table 2. this indicated that the proposed method is suitable for the assay of BOS.

Table 2: Chromatographic separation characteristics of BOS

Parameter	BOS
Number of theoretical plates (N)	3911.6
Retention time (t_R) in min	2.225
Capacity factor (k')	0.8541
Selectivity factor (α)	2.5625
Resolution (R)	-
Peak asymmetry (T)	1.05
Height equivalent to theoretical plate (HETP) in mm	0.12

ix. Linearity and regression analysis

The values of peak area of BOS were plotted against the concentration of BOS to obtain the calibration graph (Fig. 4). The calibration curve was observed to be linear and was represented by, Y = bX + c, where Y represents the peak area of BOS and X represents the concentration of BOS [18-20]. Table.3 gives the regression line, correlation coefficient, slope, intercept and % RSD. Excellent linearity was noticed in the range of 5-150 µg/ml with R^2 = 0.9998.

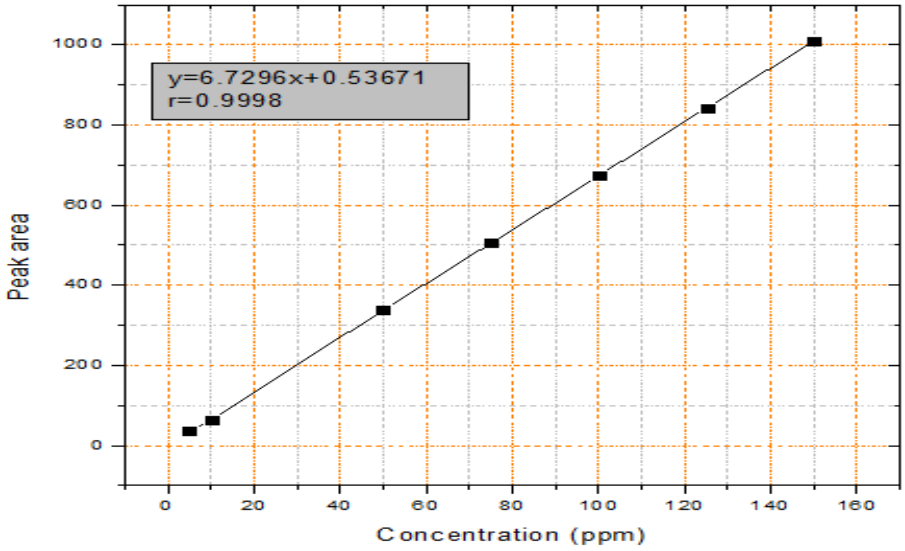

Figure 4: Beer's Law / Linearity test

Table 3: Linear-regression data for BOS

Parameter	BOS
Linear dynamic range (µg/ml)	5-150
Regression equation (Y)	Y=6.7296x+0.53671
Slope (b)	6.7296
Intercept (a)	0.5367
Correlation coefficient (R^2)	0.9998
LOD (µg/ml)	0.1121
LOQ (µg/ml)	0.3733
% RSD	0.46

x. Limits of detection and quantification ((LOD and LOQ)

Limit of detection (LOD) was established at a signal-to-noise ratio (*S*/*N*) of 3 while limit of quantification (LOQ) was established at a signal-to-noise ratio (*S*/*N*) of 10. The LOD and LOQ were calculated to be 0.1121 µg/ml and 0.3733 µg/ml respectively.

xi. Precision

Within-day (Inter-day) and between-day (Intra-day) assay precision was evaluated by determining different concentrations of pure BOS on the same day and different days. The obtained RSD values for within-day and between-day assay are shown in Table 4. The low RSD values indicated that the developed RP-HPLC method is precise.

xii. Accuracy

A standard working solution containing BOS (60, 70 and 80 µg/ml) were prepared. These standard solutions were injected six times as a test sample. From the respective peak area counts, the concentrations of the BOS were calculated using the detector responses. The accuracy, defined in terms of % bias values, was evaluated. The corresponding values are listed in Table 4. Low % bias values indicated the high accuracy of the proposed method.

Table 4: Precision and accuracy data for the assay of BOS.

Drug	Conc. of drug taken, µg/ml	Intra-day (n = 9) Conc. found, µg ml^{-1}	% Recovery	% RSD	% Bias	Inter-day (n = 9) Conc. found, µgml^{-1}	% RSD	% Bias
BOS	60	59.89	99.8	0.22	-1.0	59.96	0.32	1.0
	70	70.02	100.3	0.43	0.4	69.98	0.35	0.2
	80	79.54	99.4	0.31	-0.6	79.45	0.42	0.1

**Average of six determinations.*

xiii. Selectivity

Specificity of the proposed method was confirmed by carrying out the analysis in presence of formulation excipients. The excipients did not interfere in the determination as evident from the fact that the interfering peaks were not noticed at the retention time of BOS.

xiv. Robustness

Robustness of the method was tested by deliberate changes one at a time, in the column temperature (± 5°C), wavelength (±2nm), flow rate (± 0.2 ml/min), pH (±0.2 units) of buffer and also buffer concentration.

1.2 APPLICATIONS

i. Analysis of pharmaceutical preparation

The proposed method was successfully applied for the analysis of BOS in Tracleer tablet and the corresponding results are shown in Table 5. The low values of RSD indicated high precision of the method. The % recovery of the drug was observed to be satisfactory.

Table 5: Analysis of pharmaceutical formulations and recovery studies

Formulation	Labeled, mg	Found*, mg	Reference method [21]	% RSD	% Recovery*
Tracleer®	125	124.92	123.96	0.15	99.93

**Average of nine determinations.*
[a]Hetero Ltd., India

CONCLUSION

In conclusion, we have developed a highly selective RP-HPLC method using UV detection for the determination of BOS used as nonpeptidic dual endothelin receptor antagonist (ERA) without derivatization in pharmaceutical preparations. The developed method is rapid since preparation of pharmaceutical samples prior to chromatography is relatively simple and the total chromatographic run time is 10 min. The RP-HPLC method has high recovery and excellent reproducibility. For this reason, it can be used for the determination from pharmaceutical preparations of BOS in routine quality control measurements.

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