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# RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF BOSENTAN IN BULK AND PHARMACEUTICAL FORMULATION

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## ABSTRACT

A simple, rapid and sensitive RP-HPLC method was developed and validated for estimation of Bosentan in tablet dosage form. Chromatography was carried out by using prepacked luna C18, 5µ(250 × 4.6)mm phenomex column as a stationary phase with mobile phase containing a mixture of Acetate buffer (pH 5) : Acetonitrile in the ratio of 68:32 v/v. The flow rate was 1ml/min. The eluent was monitored at 268nm and the retention time of drug is 3.68mins. Calibration curve was plotted with a range of 2-6 µg/ml for Bosentan and the correlation coefficient was found to be 0.999. The developed method was validated in terms of linearity, precision, accuracy, specificity, limit of quantification and limit of detection. The accuracy range was found to be 98-102%. The % RSD value for all validation parameters was found to be less than 2 for RP-HPLC. The developed method can be used for routine analysis of Bosentan in pharmaceutical dosage form as well as bulk was developed and validated according to ICH guidelines.

**KEYWORDS:** Bosentan, RP-HPLC, ICH guidelines.

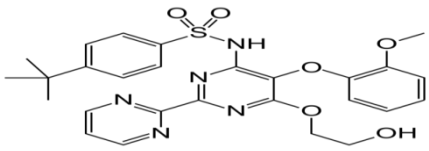
## INTRODUCTION

Bosentan is an dual endothelial receptor antagonist used in the treatment of pulmonary artery hypertension (PAH). Bosentan is a competitive antagonist of endothelin-1 at the endothelin - A and endothelin-B receptors. Bosentan is used to treat hypertension by blocking the action of endothelin in molecules that would otherwise promote narrowing of blood vessels and lead to high blood pressure.

This compound belongs to the class of organic compounds known as bipyrimidines and oligopyrimidines. These organic compounds contain two or more pyrimidine rings directlt linked to each other. Bosentan is chemically 4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]benzene-1-sulfonamide.

**Category:** Endothelial receptor antagonist.

**Classification:** Anti-hypertensive.



Bosentan is poorly soluble in water, solubility increases at higher pH.

## MATERIALS AND METHODS

### Chemicals and Reagents

Pure drug sample of Bosentan was obtained from MSN laboratories and tablet formulation (BOSENTAS) was purchased from Medplus, Hyderabad, India, with labelclaim of 62.5mg.

Acetonitrile, methanol, HPLC grade water, glacial acetic acid was procured from SD-Fine Chem-Limited and sodium acetate was procured from MERCK.

### Instrumentation

The analysis was performed by using Schimazdu 20AD RP-HPLC instrument with Phenomenex Luna C18 column (250×4.6mm) 5µm, it contains Rhenodyne valve with 20µl fixed loop injector with UV –Visible detector and UV-Visible Spectrophotometry instrument i.e, Elico 210 with spectra treats software, analytical balance (Contech) are used for weighing, pH (Elico), Sonicator (Labotech) was used for degassing the mobile phase, were used during the study.

### Chromatographic Conditions

RP-HPLC analysis was carried out on C18 (Phenomenex, 250 × 4.6mm, particle size 5µm) with reversed phase column. The mobile phase consists of a mixture of acetonitrile :sodium acetate at pH 5(adjusted with glacial acetic acid) (68:32v/v). The flow rate of mobile phase

was 1ml/min with isocratic elution, the injection volume 20µl and run time was 10mins. The detection was carried out at 268nm.

**Preparation of pH 5 Sodium acetate Buffer**

Measured accurately 13.6gms og Sodium acetate was transferred into 1000ml volumetric flask and to this add 6ml of glacial acetic acid in sufficient water, adjust pH to 5 and make up the volume to 1000ml. Filter through 0.45µ membrane filter.

**Preparation of Mobile phase**

Add 320 volume of pH 5.0 acetate buffer and 680 volume of Acetonitrile and degas it.

**Preparation of Bosentan Standard stock solution**

Accurately weigh and transfer 5mg Bosentan into 5ml volumetric flask, to that mixture add 3ml diluent and sonicate to dissolve, and then make up the volume by using diluent. (1000ug/ml).

Pipette 0.5ml from 1000µg/ml into 5ml volumetric flask and make up to mark with diluent. (100ug/ml).

**Preparation of Sample Solution**

Accurately weighed 10 tablets and average weight was calculated, accurately weighed and transferred the sample equivalent to 5mg of Bosentan into 5ml volumetric flask. To this add 3ml of diluents and sonicate to dissolve it completely and make up to mark with diluent. Further pipette out 0.5ml from 1000µg/ml into 5ml volumetric flask and make up to the mark with diluent i.e., 100µg/ml.

**Selection of Analytical Wavelength**

5µg/ml solution was scanned in the wavelength range of 200-400nm in order to observe maximum absorbance. The λmax for Bosentan is 268nm, since it shows maximum absorbance at λmax.


**Fig. 1: Scanned spectrum of Bosentan.**

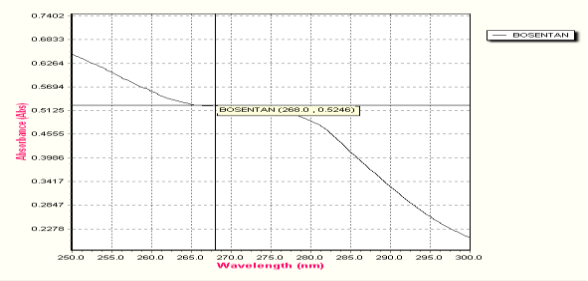



**Fig. 2: Scanned spectrum of Bosentan.**

**Optimization of proposedmethod**

**Trail 1:**

**Diluents** **:** Mobile phase

**Column** **:** C18 phenomenex

**Mobile phase** **:** pH 5.0 Citrophosphate Buffer: Acetonitrile (70:30)

**Injection volume** **:** 20µl

**Flow rate** **:** 1ml/min

**Detection wavelength** **:** 268nm

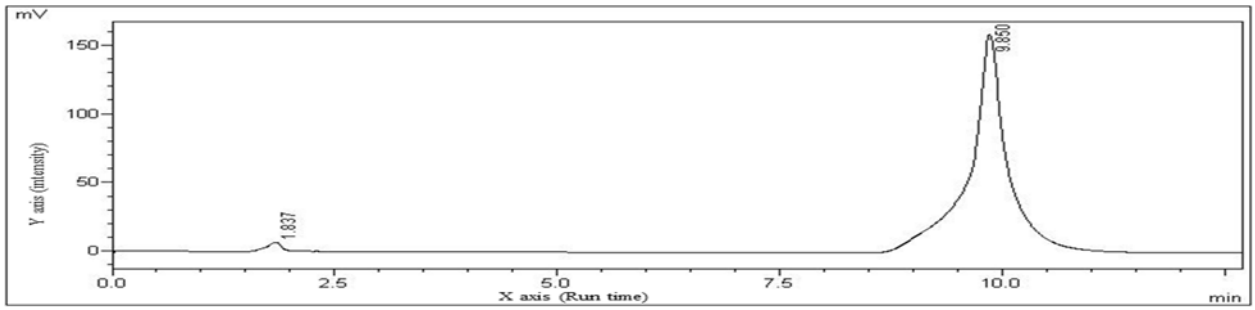



**Fig. 3: Chromatogram of trail 1.**

| Name | RT (mins) | Area | Theoretical plates | Tailing factor | Resolution |
|---|---|---|---|---|---|
| Bosentan | 9.8 | 4392591 | 5951.228 | 0.85 | 20.6 |

**Trail 2:**

**Diluents** : Mobile phase

**Column** : C18 phenomenex

**Mobile phase** : pH 3.7, Acetate Buffer : Acetonitrile ( 50:50)

**Injection volume** : 20µl

**Flow rate** : 1ml/min

**Detection wavelength** : 268nm

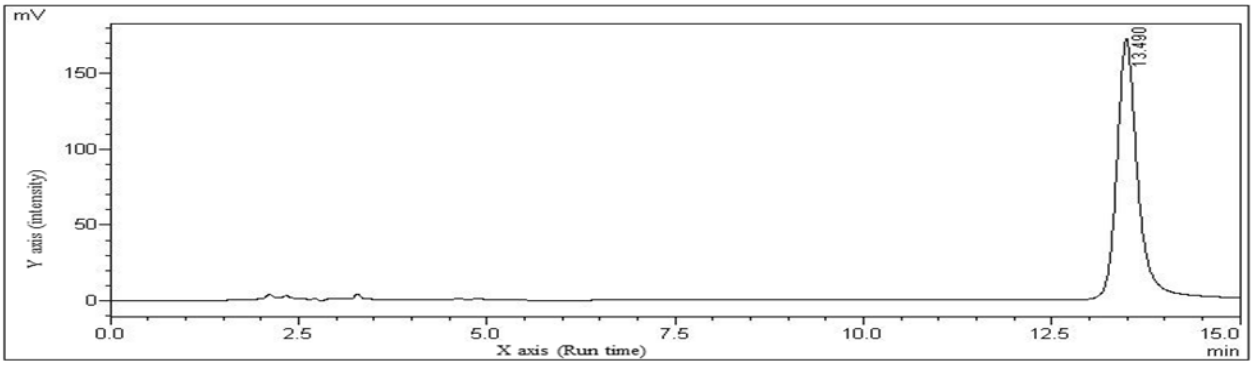



**Fig. 4: Chromatogram of trail 2.**

| Name | RT (mins) | Area | Theoretical plates | Tailing factor | Resolution |
|---|---|---|---|---|---|
| Bosentan | 13.49 | 3008599 | 13517.054 | 1.1 | 9.8 |

**Trail 3:**

**Diluents** : Mobile phase

**Column** : C18 phenomenex

**Mobile phase** : pH 3.7, Acetate Buffer : Acetonitrile ( 40:60)

**Injection volume** : 20µl

**Flow rate** : 1ml/min

**Detection wavelength** : 268nm

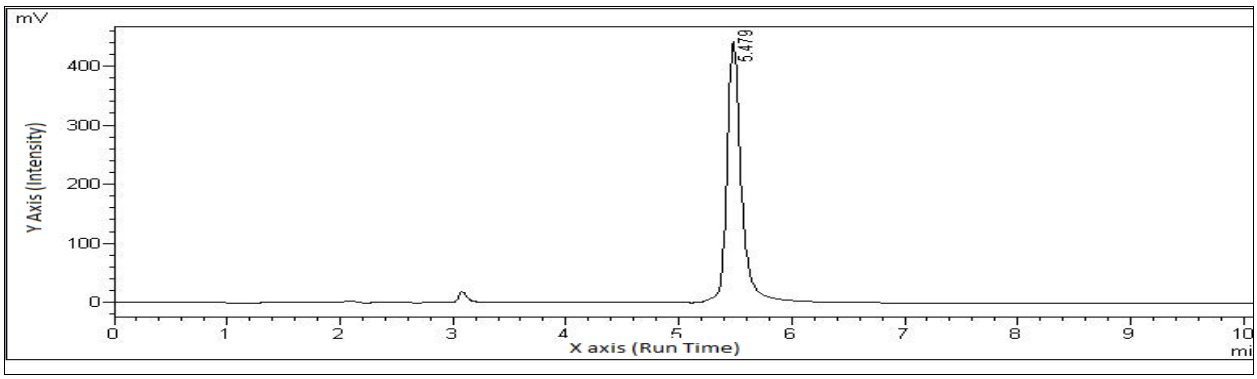



**Fig. 5: Chromatogram of trail 3.**

| Name | RT (mins) | Area | Theoretical plates | Tailing factor | Resolution |
|---|---|---|---|---|---|
| Bosentan | 5.47 | 377530 | 1889 | 1.33 | 20.2 |

**Trail 4:**

**Diluents** : Mobile phase

**Column** : C18 phenomenex

**Mobile phase** : pH 3.7, Acetate Buffer : Acetonitrile ( 30:70)

**Injection volume** : 20µl

**Flow rate** : 1ml/min

**Detection wavelength** : 268nm

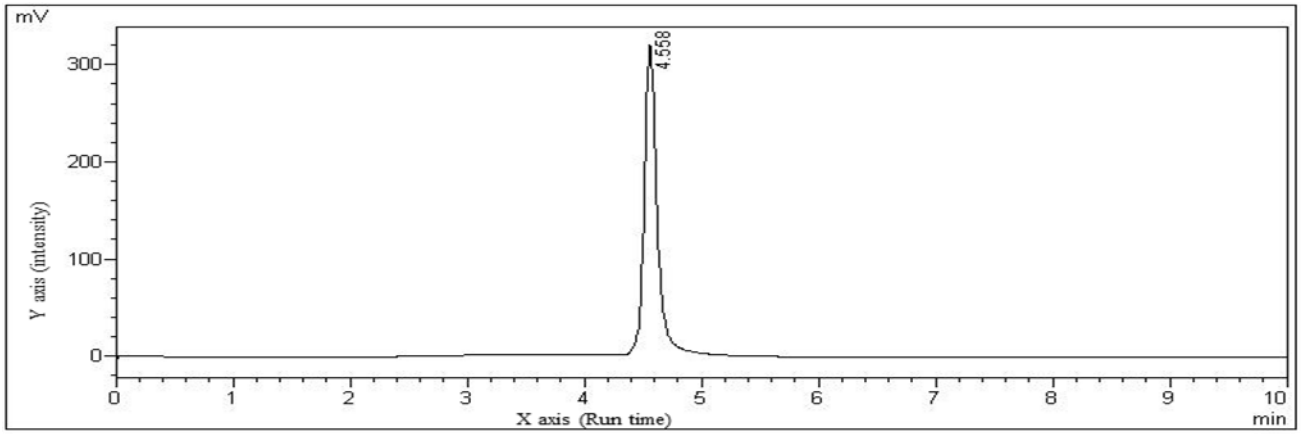



**Fig. 6: Chromatogram of trail 4.**

| Name | RT (mins) | Area | Theoretical plates | Tailing factor | Resolution |
|---|---|---|---|---|---|
| Bosentan | 4.56 | 224948 | 1876 | 1.23 | 20.2 |

**Trail 5:**

**Diluents** : Mobile phase

**Column** : C18 phenomenex

**Mobile phase** : pH 5, Acetate Buffer : Acetonitrile ( 50:50)

**Injection volume** : 20µl

**Flow rate** : 1ml/min

**Detection wavelength** : 268nm

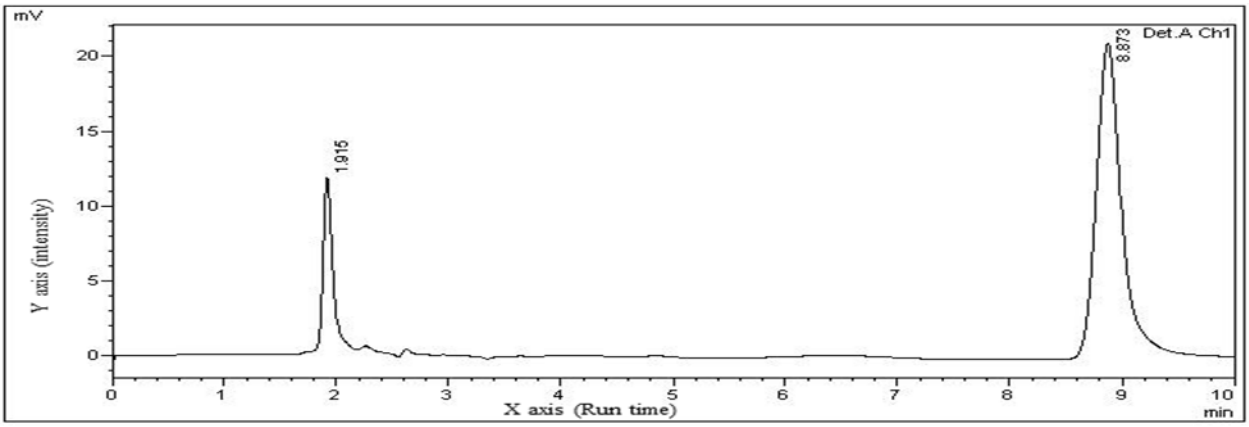



**Fig. 7: Chromatogram of trail 5.**

| Name | RT (mins) | Area | Theoretical plates | Tailing factor | Resolution |
|---|---|---|---|---|---|
| Bosentan | 8.8 | 320716 | 8269.1 | 1.33 | 20.2 |

**Trail 6:**

**Diluents** : Mobile phase

**Column** : C18 phenomenex

**Mobile phase** : pH 5, Acetate Buffer : Acetonitrile ( 40:60)

**Injection volume** : 20µl

**Flow rate** : 1ml/min

**Detection wavelength** : 268nm

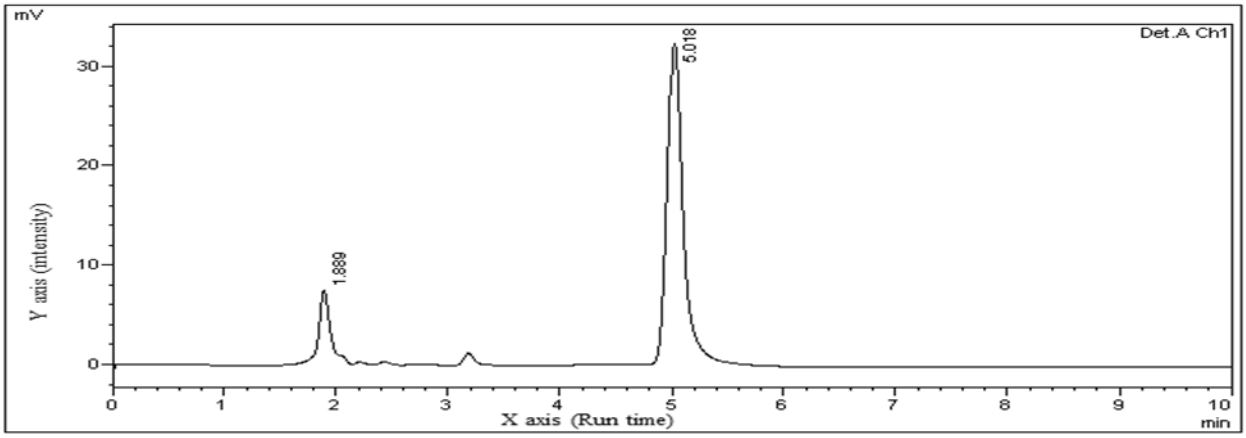



**Fig. 8: Chromatogram of trail 6.**

| Name | RT (mins) | Area | Theoretical plates | Tailing factor | Resolution |
|---|---|---|---|---|---|
| Bosentan | 5.01 | 306936 | 6551.4 | 1.4 | 16.5 |

## CONCLUSION

Trail 1: More column wash is required for the column to get stabilized

Trail 2: Here the drug is eluted slowly

Trail 3: Theoretical plate count is below 2000

Trail 4: Theoretical plate count is below 2000

Trail 5: Here the drug is eluted slowly

Trail 6: To decrease the retention time

Trail 7: Optimized method

**Optimized method**

**Diluent** : Mobile phase

**Column** : Phenomenex (250 × 4.6mm) 5µ

**Mobile phase** : (pH 5)acetate buffer: acetonitrile (32:68)

**Injection Volume** : 20µl

**Flow rate** : 1.0ml/min

**Detection wavelength** : 271nm

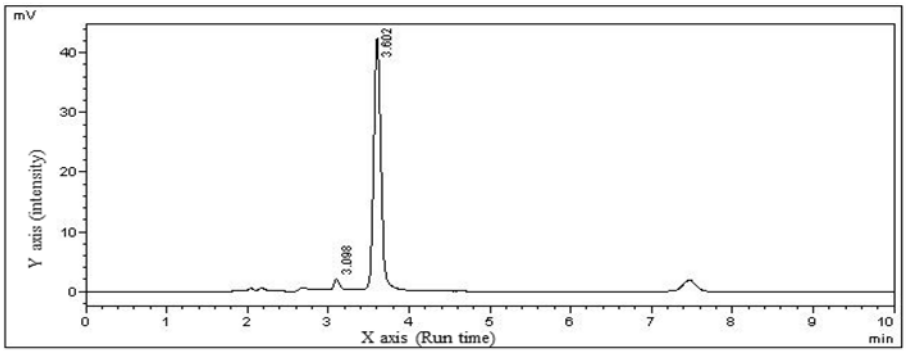



**Fig. Chromatogram of trial 7.**

| Name | RT(mins) | Area | Theoretical plate | Tailing factor | Resolution |
|---|---|---|---|---|---|
| **Bosentan** | **3.69** | **340264** | **6872.5** | **1.2** | **10.3** |

**Conclusion:** Peak shape is symmetrical and retention time is less and all parameters are within limits.

**Method Validation**

RP-HPLC method was developed and validated by using following parameters such as linearity, precision. Accuracy, robustness, LOD, LOQ.

**System Suitability**

In HPLC, it is an integral part of method development and to ensure the performance of HPLC system. The parameters such as retention time (RT), number of theoretical plates (N), and tailing factor (T) were evaluated for six replicate injections at a concentration of 3µg/ml.

**Linearity**

Linearity of an analytical procedure is its "ability (within a given range) to obtain test results which are directly proportional the concentration (amount) of analyte in the sample".

**Accuracy**

ICH defines the accuracy of an analytical procedure as **"**the closeness of agreement between the conventional true value or an accepted reference value and the value found**".**

A study of recovery was conducted from about 80%, 100% and 120% of the initial assay concentration.

**Precision**

The precision of an analytical procedure "expresses the closeness of agreement between a series of measurement obtained from multiple sampling from the same homogenous sample under the prescribed conditions". Precision of an analytical procedure is usually expressed the variance standard deviation of coefficient of variation of a series of measurement.

- **Intraday Precision**

Repeatability expresses "the precision under the same operating conditions over a short interval of time". Repeatability is also termed intra-assay precision.

- **Interday Precision**

The same sample prepared is checked on the next day.

**Limit of Detection (LOD) and Limit of Quantification (LOQ)**

Limit of detection is the **"**lowest concentration of analyte in a sample which can be detected, but not necessarily quantitated, as an exact value under the stated**".**

N be quantitatively determined with Limit of quantification is the **"**lowest concentration of analyte in a sample which can be determined with suitable precision and accuracy**"**

LOD and LOQ of drug were calculated using the following equations designated by international Conference on Harmonization (ICH)guidelines.

LOD =3.3 x σ/S

LOQ= 10 x σ/S

Where σ is the standard deviation of response

S is slope of the calibration curve

**Robustness**

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

## RESULTS AND DISCUSSION

### Results of RP-HPLC

**System Suitability**

The six replicates of injections at a concentration of 3µg/ml were prepared separately and injected and all the parameters were calculated.

**Table 1: Results of System Suitability.**

| S.NO | CONC (ug/ml) | R.T (mins) | AREA | Theoretical Plates | Tailing Factor |
|---|---|---|---|---|---|
| 1 | 3 | 3.61 | 121245 | 7550.1 | 1.177 |
| 2 | 3 | 3.61 | 122453 | 7533.25 | 1.185 |
| 3 | 3 | 3.61 | 121527 | 7554.35 | 1.177 |
| 4 | 3 | 3.61 | 122144 | 7539.27 | 1.182 |
| 5 | 3 | 3.61 | 122530 | 7879.92 | 1.168 |
| 6 | 3 | 3.61 | 122068 | 7900.25 | 1.157 |

**Specificity**

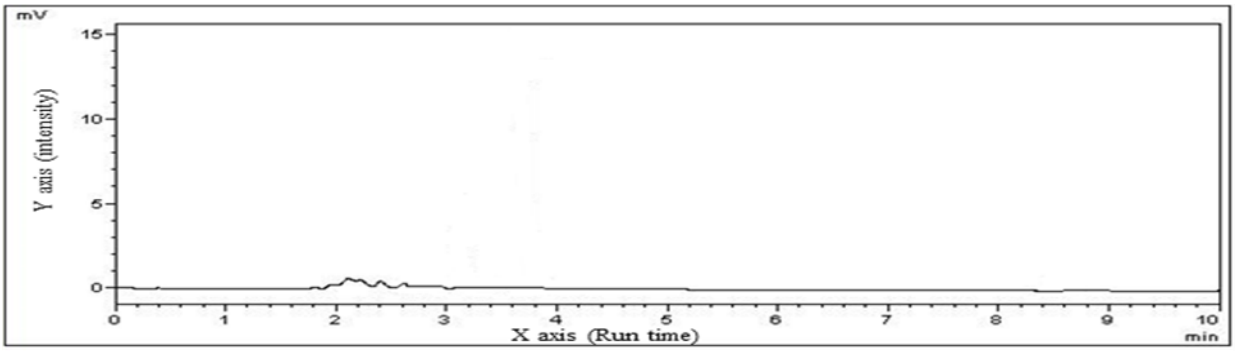



**Fig. Chromatogram of Blank.**

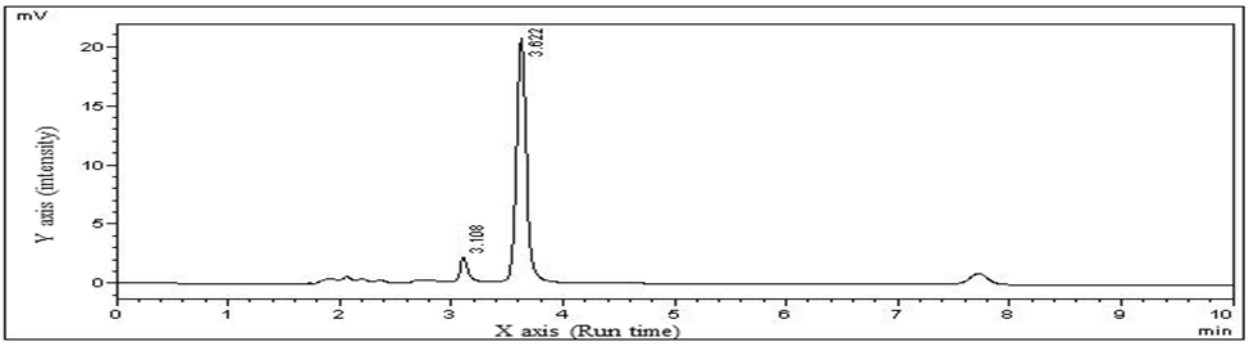



**Fig. Chromatogram of Standard 3µg/ml.**

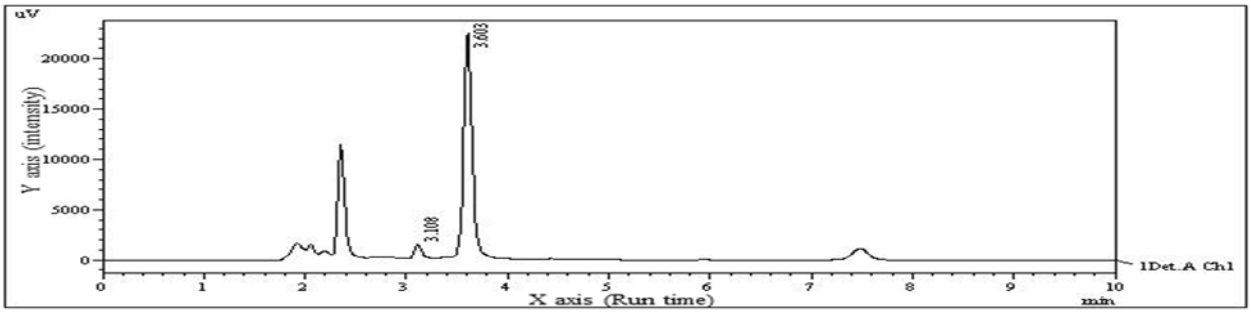



**Fig. Chromatogram of Sample 3µg/ml.**

## Linearity

The linearity was determined using standard solution, to establish linearity following concentration range of 2, 3, 4, 5, 6µg/ml.

**Table 2: Results of Linearity.**

| CONC (µg/ml) | RT (mins) | PEAK AREA |
|---|---|---|
| 2 | 3.68 | 84910 |
| 3 | 3.68 | 121527 |
| 4 | 3.69 | 159843 |
| 5 | 3.68 | 195189 |
| 6 | 3.69 | 229548 |

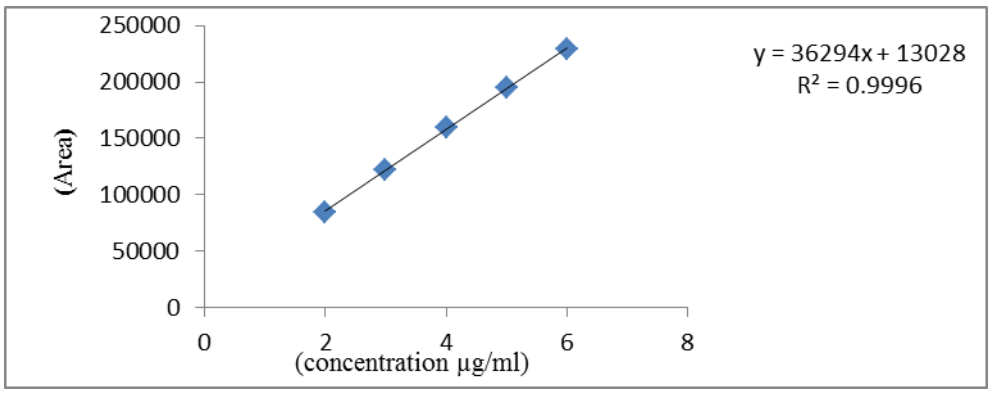



**Fig. Linearity graph of RP-HPLC.**

**Accuracy**

The accuracy was developed by recovery studies which were carried out at three different spiked levels i.e., 80%, 100%, 120%.

**Table 3: Results of Accuracy.**

| Accuracy level % | Amount pure added (µg/ml) | Amount of sample added (µg/ml) | Total concentration (µg/ml) | Area | % Recovery | Avg % Recovery | Mean | S.D | %RSD |
|---|---|---|---|---|---|---|---|---|---|
| 80 | 1.2 | 1.5 | 2.7 | 109880 | 100.4 | 100.03 | 97648.7 | 489.8 | 0.5 |
| | 1.2 | 1.5 | 2.7 | 109086 | 99.7 | | | | |
| | 1.2 | 1.5 | 2.7 | 109570 | 100.2 | | | | |
| 100 | 1.5 | 1.5 | 3.0 | 121475 | 99.9 | 99.8 | 121638.7 | 350.4 | 0.29 |
| | 1.5 | 1.5 | 3.0 | 121041 | 99.6 | | | | |
| | 1.5 | 1.5 | 3.0 | 121400 | 99.9 | | | | |
| 120 | 1.8 | 1.5 | 3.3 | 133422 | 99.8 | 99.5 | 145160.3 | 777.2 | 0.54 |
| | 1.8 | 1.5 | 3.3 | 134083 | 100.3 | | | | |
| | 1.8 | 1.5 | 3.3 | 134843 | 100.8 | | | | |

**Precision**

**Table 4: Intraday Precision.**

| S.NO | RETENTION TIME(mins) | AREA | %ASSAY |
|---|---|---|---|
| 1 | 3.648 | 122895 | 99.5 |
| 2 | 3.603 | 122963 | 99.5 |
| 3 | 3.645 | 122898 | 99.5 |
| 4 | 3.603 | 122842 | 99.5 |
| 5 | 3.643 | 123140 | 99.7 |
| 6 | 3.648 | 122804 | 99.4 |
| | **MEAN** | 122923 | 99.5 |
| | **S.D** | 119.01 | 0.09 |
| | **%RSD** | 0.1 | 0.1 |

**Table 5: Inter Day Precision.**

| S.NO | RETENTION TIME(mins) | AREA | %ASSAY |
|---|---|---|---|
| 1 | 3.648 | 123052 | 99.6 |
| 2 | 3.642 | 122996 | 99.6 |
| 3 | 3.645 | 123075 | 99.7 |
| 4 | 3.645 | 123236 | 99.8 |
| 5 | 3.643 | 123253 | 99.8 |
| 6 | 3.642 | 122729 | 99.4 |
| | **MEAN** | 123056.83 | 99.65 |
| | **S.D** | 174.22 | 0.15 |
| | **%RSD** | 0.14 | 0.15 |

**Robustness**

**Change in Flow rate (± 0.2ml/min)**

**Table 6: Results of Change in Flow rate.**

| Parameter | Flow Rate-0.8ml/min | | | | Flow Rate-1.2ml/min | | | |
|---|---|---|---|---|---|---|---|---|
| | Conc | R.T | Area | %Assay | Conc | R.T | Area | %Assay |
| 1 | 3µg/ml | 3.8mins | 123086 | 99.7 | 3µg/ml | 3.5mins | 123032 | 99.6 |
| 2 | 3µg/ml | 3.7mins | 122993 | 99.6 | 3µg/ml | 3.5mins | 123229 | 99.8 |
| 3 | 3µg/ml | 3.8mins | 123090 | 99.7 | 3µg/ml | 3.5mins | 123182 | 99.8 |
| Mean | | | 123056.33 | 99.66 | | | 123148 | 99.7 |
| S.D | | | 44.81 | 0.06 | | | 84.01 | 0.115 |
| %RSD | | | 0.04 | 0.06 | | | 0.07 | 0.12 |

**Change in Mobile phase ratio (± 2)**

**Table 7: Results of Change in Mobile phase.**

| Parameter | Ratio-Buffer : ACN (34:66) | | | | Ratio-Buffer : ACN (30:70) | | | |
|---|---|---|---|---|---|---|---|---|
| | Conc | R.T | Area | %Assay | Conc | R.T | Area | %Assay |
| 1 | 3µg/ml | 3.74mins | 122739 | 99.4 | 3µg/ml | 3.5mins | 123299 | 99.8 |
| 2 | 3µg/ml | 3.74mins | 123002 | 99.6 | 3µg/ml | 3.5mins | 123270 | 99.9 |
| 3 | 3µg/ml | 3.74mins | 123217 | 99.8 | 3µg/ml | 3.5mins | 123137 | 99.7 |
| Mean | | | 122986 | 99.6 | | | 123235.3 | 99.8 |
| S.D | | | 239.4 | 0.2 | | | 86.4 | 0.1 |
| %RSD | | | 0.19 | 0.2 | | | 0.07 | 0.1 |

**Change in pH (± 0.2)**

**Table 8: Results of Change in pH.**

| Parameter | pH of Buffer – 4.95 | | | | pH of Buffer – 5.05 | | | |
|---|---|---|---|---|---|---|---|---|
| | Conc | R.T | Area | %Assay | Conc | R.T | Area | %Assay |
| 1 | 3µg/ml | 3.516mins | 123080 | 99.7 | 3µg/ml | 3.749mins | 123002 | 99.6 |
| 2 | 3µg/ml | 3.516mins | 122799 | 99.4 | 3µg/ml | 3.749mins | 122739 | 99.4 |
| 3 | 3µg/ml | 3.516mins | 122713 | 99.4 | 3µg/ml | 3.749mins | 123217 | 99.8 |
| Mean | | | 122864 | 99.5 | | | 122986 | 99.6 |
| S.D | | | 191.94 | 0.173 | | | 239.4 | 0.2 |
| %RSD | | | 0.16 | 0.17 | | | 0.19 | 0.2 |

**LOD & LOQ**

**Table 9: Results of LOD & LOQ.**

| S.NO | SAMPLE | INTERCEPT | SLOPE | LOD | LOQ |
|---|---|---|---|---|---|
| 1 | 3µg/ml | 13237 | 36597 | $3.3 \times \sigma/S$ $=3.3 \times 1339.79/36299$ $=0.12$ | $10\times \sigma/S$ $=10 \times 1339.79/36299$ $=0.37$ |
| 2 | | 15446 | 36007 | | |
| 3 | | 13028 | 36294 | | |
| | MEAN | 13903.67 | 36299 | | |
| | SD | 1339.79 | 295.04 | | |

**Assay of Marketed Formulation**

**Table 10: Results of Assay.**

| Dosage form | Labeled claim | Amount found | % Recovery | % RSD |
|---|---|---|---|---|
| Tablet Bosentas | 62.5mg | 62.7mg | 100.3 | 0.26% |
| | | 62.9mg | 100.7 | |
| | | 62.6mg | 100.2 | |

## CONCLUSION

The method was successfully developed for estimation of Bosentan in pharmaceutical dosage form by using RP-HPLC and the validated parameters results have proved that the method is selective, precise, accurate and linear.

The developed method was validated as per the International Conference On Harmonization ICH(Q2B)guidelines, and was found to be applicable for routine quantitative analysis of Bosentan by RP-HPLC in tablet dosage forms.

This method is more sensitive than previously reported methods, due to its high sensitivity. Hence ablove method can be used in quality control for routine analysis of tablets of Bosentan without any interference.

We can extend this work by performing degradation studies and simultaneous method. we can still reduce the retention time. With high sensitive instrument we can reduce concentration to ngs. Even LC-MS analysis can also be done with same buffer since the buffer is suitable for LC-MS.

## ACKNOWLEDGEMENT

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