

**BIOANALYTICAL METHOD DEVELOPMENT, VALIDATION AND  
QUANTIFICATION OF BOSENTAN BY HIGH-PERFORMANCE  
LIQUID CHROMATOGRAPHY IN RAT PLASMA****Nallakumar P.\*<sup>1</sup> and Siva Kumar R.<sup>2</sup>**

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**ABSTRACT**

A Simple and rapid bioanalytical high performance liquid chromatographic (HPLC) method for the determination of Bosentan using Losertan as an internal standard was developed and validated as per regulatory requirements. Sample preparation was accomplished through liquid phase extraction and chromatographic separation on a reverse phase column. The mobile phase consists of mixture of methanol and water in the ratio of 50:50 at a flow rate of 1ml/min. The wavelength used for the detection of bosentan was 225nm with a total run time of 6minutes. The retention times of bosentan and losertan were found to be 2 and 4 respectively. The method was developed and tested for the linearity range of 250-750ng/ml. The method was validated for accuracy, precision, linearity and recovery in compliance

to international regulatory guidelines.

**KEYWORDS:** Bosten, HPLC, Validation.

**Abbreviations:** CV: Coefficient Variation; HPLC: High Performance Liquid Chromatography; IS: Internal Standard; LC: Liquid Chromatography; LLOQ: Lower Limit of Quantitation; LQC: Low Quality Control; MQC: Medium Quality Control; HQC: High Quality Control; PK: Pharmacokinetics; QC: Quality Control; RP: Reverse Phase, UV: Ultraviolet spectrophotometry; C<sub>max</sub>: the maximum plasma concentration of the drug.

## INTRODUCTION

Bosentan, 4-*t*-butyl-N-(6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2,2-bipyrimidin-4-yl)benzenesulfonamide, is an endothelial receptor antagonist used in the treatment of pulmonary artery hypertension. Bosentan is available in the tablet dosage form. Hypertension also known as high blood pressure is a long term medical condition in which the blood pressure in the arteries is persistently elevated. High blood pressure usually does not cause symptoms. **Endothelins** are peptides that constrict blood vessels and raise blood pressure. They are normally kept in balance by other mechanisms, but when they are over-expressed, they contribute to high blood pressure (hypertension) and heart disease.

Endothelins are 21-amino acid vasoconstricting peptides produced primarily in the endothelium having a key role in vascular homeostasis. Endothelins are implicated in vascular diseases of several organ systems, including the heart, general circulation and brain.

Literature survey has revealed that there are only a few methods reported for the determination of BOSENTAN in plasma by liquid chromatography. Methods reported in the literature for the estimation of bosentan in bulk and biological fluids include A rapid high-performance liquid chromatographic bioanalytical method development and validation for Bosentan in human plasma by taking 70% ammonium acetate & 30% acetonitrile as a mobile phase and Phenomenex luna C18 as column at 5.7-7.8mins as retention time. Development, estimation and validation of Bosentan in bulk and in its pharmaceutical formulation by UV-Vis Spectroscopic method using Methanol:Water as mobile phase.

From the literature survey, Various analytical method developments have been reported for the estimation of bosentan by RP-HPLC and UV-methods. Here an attempt was made to develop the bio-analytical method for the estimation of bosentan from plasma using losartan as internal standard and to validate as per international regulatory guidelines. Runtime was decreased while developing the method.

## MATERIALS AND METHODS

### Chemicals and reagents

Bosentan (Figure 1) of the highest quality has been purchased from sigma Aldrich (Mumbai, India) and Losartan (Internal Standard) (Figure 1) was kindly donated by Torrent Pharmaceuticals limited (Ahmedabad, India). HPLC grade Methanol 99.8% was obtained

from Merck chemicals (Bangalore, India). Potassium dihydrogen phosphate (AR Grade), Dipotassium hydrogen phosphate (AR Grade).

Chemicals	Make
water	HPLC grade
Acetonitrile	Merck(HPLC grade)
Methanol	Merck(HPLC grade)
Potassium dihydrogen phosphate	AR grade
Dipotassium hydrogen phosphate	AR grade

### Instrumentation

RP-HPLC analysis was performed on Applied Biosystems is enhanced by the high degree of automation and data processing capabilities of Analyst software supplied by Labindia Instrument Pvt. Ltd. (Gurgaon, India). The LC part consists of Shimadzu auto sampler LC-10 series chromatographic system (Shimadzu Corporation, Kyoto, Japan) equipped with dual pump (LC10AT-VP). The column oven employed (CTO-10AS VP) and vacuum suction pump was Varian HS 602 Vacuum Pump supplied by Agilent Technologies India Pvt. Ltd. (Haryana, India).

### Chromatographic condition

LC (2010)AHT with the C18 column (4.6X250mm, 5 $\mu$ m), (X bridge C18 5 $\mu$ m) was used and the wave length used for the detection was 254nm. The other method conditions included were the column oven temperature of 23°C, flow rate of 1ml/min, run time of 6min and injection volume of 5 $\mu$ l.

**Table 1: HPLC chromatographic condition.**

<b>Column</b>	<b>Eclipse XDB - Phenyl 4.6mm LD X 250mm(5<math>\mu</math>m)</b>
<b>Flow rate</b>	<b>1.0 ml/min</b>
<b>Temperature</b>	<b>Ambient (23 c)</b>
<b>Detector</b>	<b>UV (254 nm)</b>
<b>Sample volume</b>	<b>5 <math>\mu</math>l</b>

### Preparation of stock and standard solutions

The Bosentan standard was weighed twice separately and prepared separately using methanol and water (50:50) to yield two primary standard stock solutions (1 and 2) with a concentration of 50  $\mu$ g/mL. Secondary and working stock solutions for the calibration curve were prepared from bosentan primary stock solution-1 by methanol and water (50:50). The

quality controls working stock solution were prepared from primary stock solution-2 by methanol and water (50:50). These working stock solutions (1 and 2) were further diluted to obtain bosentan calibration spiking stock solution for the final concentrations of 50,80,100,120 and 150 ng/mL and concentrations of (250, 500 and 750 ng/mL) quality control spiking stock solutions were prepared. The internal standard (Losertan) was weighed and prepared separately using methanol and water (50:50) to obtain a primary stock solution of 50 $\mu$  mg/mL. The working internal standard solution with a concentration of 500 ng/mL was prepared by diluting the primary stock solution with methanol.

### **Extraction of Bosentan from rat plasma**

Extraction of Bosentan from rat plasma sample was carried out by using simple liquid-liquid extraction method. 0.2ml from each concentration level and 0.1ml of IS are transferred into 10ml volumetric flask and make up the volume. Shake for 10 minutes. From the above sample collect 0.25ml into centrifuge tube. Add 3ml of ethyl acetate. Shake for 15 minutes. Centrifuge for 20 minutes & collect the supernatant layer and evaporate. Reconstitute the tubes with 0.25ml mobile phase. The representative chromatograms were shown in Figure 2.

### **Bioanalytical method validation**

Preparation of calibration curve: The linearity of the method was evaluated by a calibration curve in the range of 0.98 – 998 ng/mL of metaxalone, including lower limit of Quantitation (LLOQ). The calibration curve was achieved by plotting the peak area ratios of metaxalone and internal standard versus the concentration of metaxalone by least-squares linear regression analysis. The calibration curve requires a correlation coefficient ( $R^2$ ) of >0.99. The acceptance criteria for each back-calculated standard concentration should be within 15% of the nominal concentration, except it should not exceed 20% for the LLOQ. Each validation run consisted of a double blank, system suitability sample, a zero standard, calibration curve consisting of ten non-zero samples covering the total range (0.98 – 998 ng/mL) and QC samples at three concentrations ( $N = 6$ , at each concentration). Such validation runs were generated on six consecutive days.

### **Accuracy and precision**

Intra-day and Inter-day accuracy and precision were determined by duplicate analysis of six sets of samples spiked with four different concentrations of bosentan at low, medium, high quality control samples (200, 500 and 750 ng/mL) including LLOQ (200 ng/mL) within a day or on 6 consecutive days. For acceptance criteria for intra and inter-day precision, accuracy

should be within 85–115% of the nominal concentration and coefficient of variation (%CV) values should be <15% over the calibration range, except at the LLOQ, where accuracy should be between 80 – 120% and %CV should not be more than 20%.

**Selectivity:** The selectivity of the assay methodology was established using a minimum of six independent sources of the same matrix. There were no interferences from the endogenous material at the retention time for both Bosentan and internal standard (Losertan). The representative chromatogram is shown in Figure 3.

**Recovery:** Recovery of bosentan was evaluated by comparing the mean peak areas of three extracted low, medium and high quality control samples to mean peak areas of three neat reference solutions (un-extracted). Recovery of internal standard was evaluated at a concentration of 200 ng/mL and corresponding mean peak area of the extracted samples compared to the mean peak areas of neat reference solutions. Recovery of the analyte need not be 100%, but the extent of recovery for analyte (Bosentan) and internal standard (Losertan) should be consistent and reproducible.

**Stability:** In order to find out the stability of bosentan in rat plasma, bench top stability, freeze thaw stability, auto injector stability and long term stability studies were carried out by using six replicates of the low and high plasma quality control samples. For the bench top stability, frozen plasma samples were kept at room temperature for 24 hr before sample preparation. Freeze–thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature for 2–3 hr and refrozen for 12–24 hr for each cycle. Auto sampler stability of metaxalone was tested by processed and reconstituted low and high plasma QC samples, which were injected 24 hr after reconstitution and were compared with freshly prepared QC samples. Long term stability of metaxalone in rat plasma was tested after storage at approximately –70°C for 30 days. For the acceptance criteria of stability, the deviation compared to the freshly prepared standard should be within  $\pm 15\%$  of the nominal concentration.

**Matrix effect:** The matrix effect was performed in 6 different lots of rat plasma by taking 47.5  $\mu$ L of rat plasma and 2.5  $\mu$ L of methanol: water (50:50) solution. From the mixture, take 25  $\mu$ L and add 375  $\mu$ L of blank methanol containing. This blank mixture was vortexed for 5 min at 885 g and centrifuged at 19283 g for 8 min to prepare the extracted blank. The aqueous equivalent solution was prepared by taking 25  $\mu$ L of water and adding 25  $\mu$ L of

internal standard along with 350  $\mu$ l of methanol containing 0.1% trichloroacetic acid. The extracted blank supernatant and aqueous equivalent solution were mixed in a ratio of (1:1) solution. The blank aqueous solution was prepared by mixing 25  $\mu$ l of water along with 375  $\mu$ l of methanol and subjected to vortexing. The neat solution and post extracted solution. Were prepared by mixing aqueous equivalent solution to both extracted blank and blank aqueous solution in a ratio of (1:1) solution. Both solutions were vortexed and subjected to RP-HPLC for analysis.

Matrix suppression or enhancement was calculated as follows:  $100 \times \text{mean peak area of post extracted sample} / \text{mean peak area of neat standard solution}$ . The acceptance criteria for matrix effect implied that the %CV should be less than 15% of matrices tested and at least 80% of matrices should meet the above criteria. The results obtained were displayed in Table 2.

**Table 2: Calibration table.**

Day	R	Slope	Intercept
1	0.992	14769	510323
2	0.992	14698	510264
3	0.993	14779	510233
4	0.994	14773	510289
5	0.992	14777	510283
6	0.993	14765	510269
Mean	0.992	14777	510298
SD	8.844E-05	1.277E-06	0.1272
SE	3.452E-05	5.123E-07	0.113

**Table 3: Intraday precision and accuracy (n=6).**

Concentration added (ng/ml)	Concentration found (mean $\pm$ SD) (ng/ml)	%CV	%Bias
50	49.8 $\pm$ 0.19	1.64	-1.49
250	249.3 $\pm$ 0.33	1.32	-1.25
500	498.11 $\pm$ 1.390	1.26	-2.45
750	687.26 $\pm$ 1.993	1.84	-3.67

**Table 4: Interday Accuracy and precision (n=6).**

Concentration added (ng/ml)	Concentration found (mean $\pm$ SD) (ng/ml)	%CV	%Bias
50	49.9 $\pm$ 0.34	1.98	-1.40
250	249.84 $\pm$ 0.43	1.99	-2.31
500	490 $\pm$ 0.520	1.41	-1.90
750	754.24 $\pm$ 1.675	1.67	-3.05

**Table 5: Extraction Recovery (n=6).**

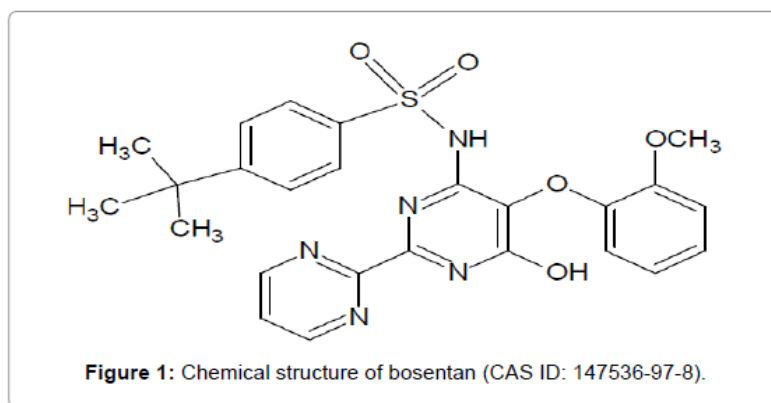
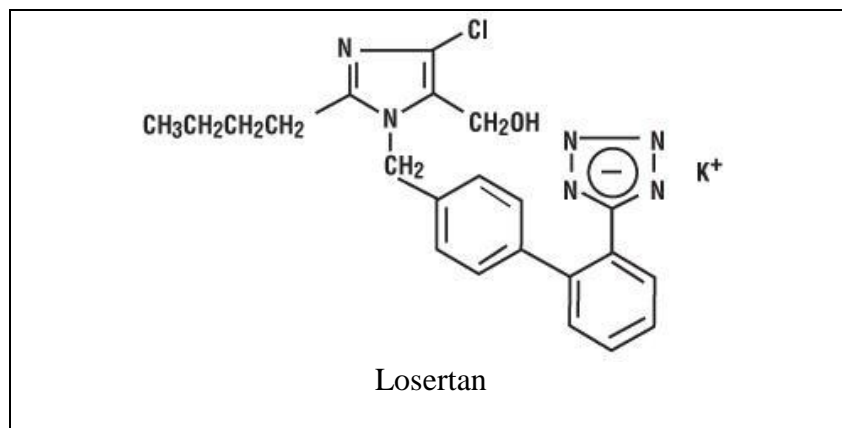
Bosentan	LQC %	MQC%	HQC%
Extraction recovery of six different aliquots of rat plasma	84.22	82.18	82.11
	83.05	83.22	83.97
	86.23	84.10	82.14
	83.13	82.19	84.12
	83.02	83.23	83.13
	83.93	84.26	84.21
Mean of extraction recovery	83.83	83.19	83.28
Losertan (IS)	83.88 ± 1.32 83.05 ± 1.29		

**Table 6: Matrix effect (n=6).**

Bosentan	LQC(ng/ml)		HQC(ng/ml)	
	Aqueous	Post Extracted	Aqueous	Post Extracted
Peak areas	6698	5832	1842799	1711811
	6782	5696	1821364	1716685
	6698	5579	1839656	1722664
	6539	5825	1813150	1725528
	6584	5867	1842968	1721779
	6736	5769	1838605	1705920
Mean peak area	6672.83	5761.33	1831987	1719693.4
Relative standard deviation	1.968627	1.618601	0.690661	0.43241655
Absolute matrix effect	90.257		93.870	

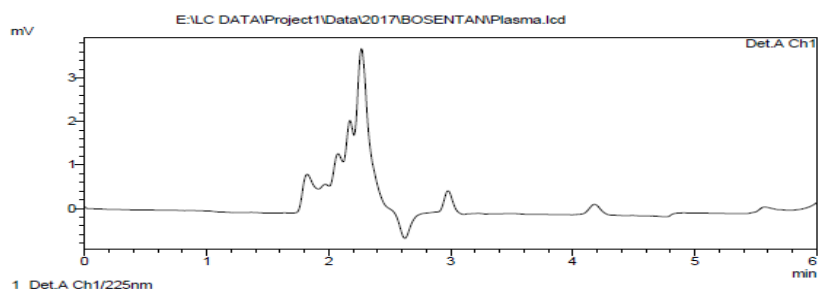
**Table 7: Stability details of Metaxalone in rat plasma sample (n=6).**

Concentration (ng/ml)	Fresh sample Concentration (ng/ml)	Sample Concentration after storage(ng/ml)	%CV	%Bias
Short term (24hr)				
200	200.3	198.63	1.92	-2.20
750	746.46	720.36	1.48	-2.59
Three Freeze thaw cycles				
200	200	198.4	1.43	-0.98
750	755.2	732.7	2.13	-2.25
Auto sampler stability (24 hr)				
200	200.6	195.4	1.36	-1.46
750.00	762.30	753.7	1.41	2.35
Long term 30 days (-85°C)				
200	199.8	192.8	2.97	-2.33
750.00	747.53	733.86	1.15	-3.70



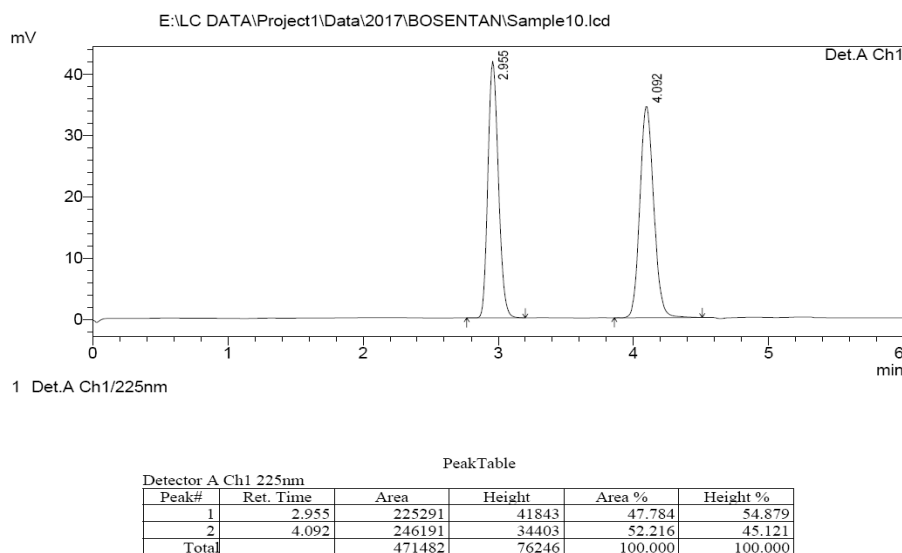
Acquired by : user1  
 Sample Name : BOSENTAN  
 Sample ID : Plasma  
 Tray# : 1  
 Vial # : 2  
 Injection Volume : 10 uL  
 Data File Name : Plasma.lcd  
 Method File Name : BOSENTAN\_P.lcm  
 Batch File Name : BOSENTAN.lcb  
 Report File Name : Default.lcr

#### <Chromatogram>

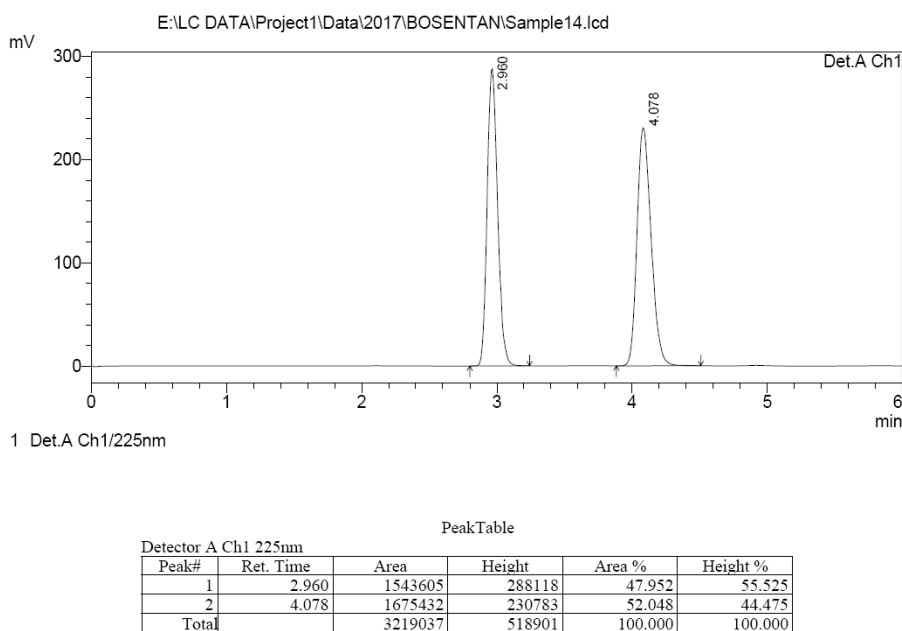


**Fig-1** Representative chromatogram of blank plasma sample.





**Fig-2 Representative chromatogram of lowest calibration standard.**



**Fig-3 Representative Chromatogram of Highest Calibration Standard.**

## RESULTS

### Selectivity and optimization of chromatographic conditions

Plasma matrices were obtained from six different sources and assayed to evaluate the selectivity of the method and the detection of interference. Metaxalone and Phenytoin

(internal standard) were well separated from the co-extracted material under the described chromatographic conditions at retention times of 2 and 4.3 min respectively. No endogenous peak from plasma was found to interfere with the elution of either the drug or the internal standard. The LLOQ which could be measured with acceptable accuracy and precision for the analyte 200 ng/mL was established (Figure 4). It indicates that the proposed method is highly selective and specific.

### Calibration curve

Linear detector response for the peak-area ratios of the metaxalone to internal standard was observed in the concentration range between 50 - 750 ng/mL with a mean correlation coefficient of 0.992. The reason for choosing a wide calibration range for Bosentan. PK study is to analyze samples of higher and lower dose concentration and different route of administration like intravenous administration and per oral where the C<sub>max</sub> concentration will be higher for intravenous. The best fit for the calibration curve could be achieved with the linear equation  $Y = MX + C$ . The mean linear regression equation of calibration curve for the analyte was  $Y = 0.14777x - 510298$ , where Y was the peak area ratio of the analyte to the IS and X was the concentration of the analyte. The results were given in the Table 3.

### Accuracy and precision

The intra-day accuracy and precision ranged between 96.2-104.11% and 1.54-1.69%, respectively. The inter-day accuracy and precision ranged from 95.56 to 110.26% and 1.38 to 1.87%, respectively. The accuracy and precision for intra and interday at the LLOQ and at LQC, MQC, HQC control samples of metaxalone in plasma were within acceptable limits (N = 6). The results of the method validation studies presented in Table 4 and 5.

### Recovery

The recovery of Bosentan in plasma was calculated at three QC levels. The response (extracted) compared to that of unextracted samples of the reference solution. The percentage recovery of Bosentan and losertan (internal standard) were found to be above 80%. Results are displayed in the Table 6.

### Stability

Stock solutions of Bosentan (5 mg/mL) and internal standard (5 mg/mL) were separately prepared. The solutions were stable for at least 1 month when stored under light-protected conditions at 4°C. The stability experiments were aimed at testing all possible conditions that

the samples might experience after collecting and prior the analysis. All stability results were summarized in Table 7. The results of three freeze– thaw cycles and bench top stability testing (24 h) when the spiked samples were kept at room temperature indicated that Bosentan was stable in rat plasma under these conditions. Analyte spiked QC samples were stable for at least 30 days if stored in the freezer at  $-85^{\circ}\text{C}$ . Testing of auto sampler stability of quality control samples indicated that Bosentan would be stable when kept in the auto sampler up to 24 h.

## DISCUSSION

A simple and selective LC method is described for the determination of BOSENTAN tablet dosage forms. Chromatographic separation was achieved on a  $\text{C}_{18}$  column using mobile phase consisting of a mixture of 50 volumes of METHANOL and 50 volumes of WATER (with detection of 225 nm. Linearity was observed in the range 50-150  $\mu\text{g}/\text{ml}$  for BOSENTAN ( $r^2 = 0.992$ ) for the amount of drugs estimated by the proposed methods was in good agreement with the label claim.

The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives. The method was found to be precise as indicated by the repeatability analysis, showing %RSD less than 2. All statistical data proves validity of the methods and can be used for routine analysis of pharmaceutical dosage form.

## CONCLUSION

In conclusion a validated RP-HPLC method has been developed for determination of BOSENTAN in the bulk and combined tablet dosage forms. The results show that the method was found to be specific, simple, accurate, precise and sensitive. The method was successfully applied for the determination of both drugs in combined tablet dosage form. In the future, this method may be applied for routine analysis of both the drugs in API and in tablet formulation.

Several analytical procedures have been proposed for the quantitative estimation of BOSENTAN separately and in combination with other drugs. To my knowledge simple, rapid analytical method for determination of BOSENTAN has not been reported so far.

So attempt was taken to develop and validate a reversed-phase high performance liquid chromatographic method for the quality control of BOSENTAN in pharmaceutical preparations with lower solvent consumption along with the short analytical run time that leads to an environmentally friendly chromatographic procedure and will allow the analysis of a large number of samples in a short period of time.

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