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A stability indicating RP-UPLC method for estimation of Bosentan and its impurities in bulk drugs and pharmaceutical dosage forms.

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ABSTRACT

This study is aimed at Developing and validating an UPLC method for Bosentan and its related substances that might coexist in bulk drugs and its tablet formulations as impurities that may originate from synthesis process or degradation. A chromatographic system consisting Waters Acquity UPLC HSS PFP, 2.1x 50mm (2.5 µm) column, mobile phase of 0.02M $KH_2PO_4$with pH 2.0 as Buffer phase and Acetonitrile: Methanol in 1:1 ratio as organic phase, with gradient elution at flow of 0.6 mL/min and UV detector set at 220 nm has shown a good chromatographic separation for Bosentan and its related substances. The developed method was validated as per ICH Guidelines and shown equivalency with API Vendor method. The developed UPLC method has run time of only 13 minutes making the method productive and may be applied for Quality control Testing.

Keywords: Bosentan, Stability indicating, RP-UPLC, Equivalency.

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INTRODUCTION

Bosentan monohydrate (4-tert-butyl-N-[6-(2-hydroxy ethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl) pyrim-idin-4-yl] benzene-1-sulfonamide monohydrate), a dual endothelin receptor antagonist (ERA) has molecular formula of C27H29N5O6 S·H2O with relative molecular mass of 569.64. It is the first orally active drug approved by United States Food and Drug Administrative as Tracleer (62.5mg and 125mg) 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]. An extensive 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 forth 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 USP Pending monograph was available(C104603). The literature survey also revealed that there was no stability-indicating RP-UPLC method for the determination of process and degradation-related impurities formed under the stress conditions in bosentan monohydrate.

Ultra performance liquid chromatography (UPLC) is a new category of separation science which builds upon well established principles of liquid chromatography, using sub 2 µm porous particles. These particles operate at elevated mobile phase velocities to produce rapid separations with increased sensitivity and increased resolution. Thus UPLC technology allows analysts time to be drastically reduced while still meeting assay acceptance criteria based on plate count, resolution and analyte retention.

In this paper we describe development and validation of related substances method for accurate quantification of six 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, and stability-indicating method for determining the process and degradation-related impurities in samples of the bosentan monohydrate bulk drug along with its validation as per USP and ICH guidelines [16-17].

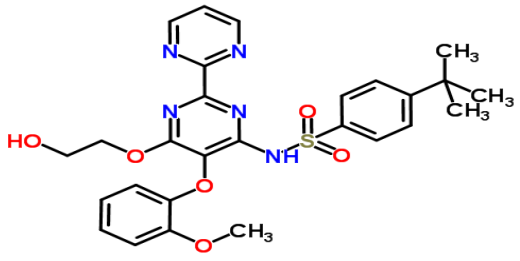

Figure1: Bosantan chemical structure

MATERIALS AND METHODS:

Instruments:

A Waters acquity ultra performance liquid chromatography equipped with PDA Detector with Binary pump. The column utilised was Acquity UPLC, HSS, PFP, 2.1x50mm, 2.5µm.

Chemicals:

All the chemicals used were of pharmaceutical grade. Acetonitrile and methanol are chromatographic grade and Potassium dihydrogen phosphate and triethyl amine AR Grade were from Merck. Bosentan and its impurities were obtained from MSN Laboratories. Bosantas tablets were obtained from cipla Ltd, India.

The compounds related to Bosantan which could be expected as impurities or might appear as degradation products have been prepared and identified by MSN Labs, Hyderabad, India.

1) Bosantan: (4-tert-butyl-N-[6-(2-hydroxy ethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl) pyrim-idin-4-yl] benzene-1-sulfonamide monohydrate).

2) Mono chloro impurity (Impurity-1): (RT-0.6) :6-chloro-5-(2-methoxy phenoxy)-2-(pyrimin-2-yl)pyrimidin-4(3H)-one.

3) Isopropyl impurity (Impurity-2): (RT-3.9):4-isopropyl-N-[6-(2-hydroxy ethoxy)-5-(2 methoxy phenoxy) [2, 2'-bi pyrimidin]-4-yl] benzene sulphonamide.

4) Pyrimidinone Impurity (Impurity-3): (RT-4.4):4-tert-butyl-N-(5,2-methoxy phenoxy)-6-oxo-2-(pyrimidin-2-yl)-1,6-dihydro pyrimidin-4-yl) benzene sulphonamide.

5) Methoxy impurity (Impurity-4): (RT-6.6): 4-tert-butyl-N-(6-methoxy-5-(2-methoxy phenoxy)-2, 2'-bi pyrimidin-4-yl] benzene sulphonamide.

6) BSN4FB impurity (Impurity-5): (RT-6.8): 4-tert-butyl-N-(6-chloro-5-(2-methoxy phenoxy)-2,2'-bi pyrimidin-4-yl]benzene sulphonamide.

7)Dimer impurity (Impurity-6): (RT-8.3): N,N'-(6,6'-(ethane-1,2,di yl bis(oxy)bis(5-(2-methoxy phenoxy)2,2'-bi pyrimidine-6,4-di yl) bis(4-tert-butyl) benzene sulphonamide.

Developing an UPLC Method:

The UPLC method carried out in this study aimed at developing chromatographic system capable of eluting and resolving Bosentan from its process related impurities and degradation products that comply with the general requirements for system suitability. Initial trials were done with 1mL of tri ethyl amine in 1L,pH adjusted to 2.5 with phosphoric acid: methanol(60:40) and acetonitrile gradient at flow rate 0.5 mL·min–1. Poor peak shape of Bosentan and resolution with impurity-2and impurity-3 was problem.

Different columns such as BEH C18, BEH C8 and different buffers such as formic acid, trifluoroacetic acid were also tried with different isocratic and gradient methods to achieve the best chromatographic separation. But long retention times and poor peak shapes were still unavoidable.

With 0.1% trifluoroacetic acid, impurity-2 and impurity-3 are co-eluting and long retention times are seen. Studied the separation and peak shape by varying pH from 2.5 to 7.0 with phosphate buffer, and observed that, as the pH is decreasing towards 3.0, peaks were strongly retaining. Also at higher pH, Impurity-2 and impurity-3 are co eluting. Added Methanol to the organic phase to study the separation on a HSS, PFP, and 50mm column at 2.0 pH. The resolution was significantly improved but peak shape was problem.

Adding tri ethyl amine to the buffer obtained better separations and peak shapes with KH2PO4 Buffer and acetonitrile and methanol as organic phase. The % of Organic phase played a key role in the retention times and resolution between impurities. After many logical trials, chromatographic condition was established such that which could be suitable for separation of drug degradation products and six known impurities. Using the optimized conditions, Bosentan and its known impurities were well separated with a resolution of greater than 1.5.

Finalized conditions:

The chromatographic column used was Acquity, UPLC, HSS, X-bridge, PFP column (50 × 2.1) mm with 2.5 µm particles. Buffer consists of a mixture of 2.72 Grams of Potassium di hydrogen phosphate with 2 mL of Tri ethyl amine pH adjusted to 2.0 using Diluted phosphoric acid. The mobile phase consists of buffer as aqueous phase and Acetonitrile: methanol at 1:1 ratio as organic phase with the gradient programme (Table-1). The flow rate of the mobile phase was 0.6 mL·min–1. The column temperature was maintained at 45°C and the detection was monitored at a wavelength of 220 nm. The injection volume was 2µL. Buffer and acetonitrile in 1:9 ratios was used as diluent. The concentration is 1000 ppm for impurities and 100 ppm for Assay method.

Table 1: Gradient programme

Time(min)	Flow(mL/min)	%A	%B
0.01	0.6	60	40
5.00	0.6	60	40
7.00	0.6	40	60
9.00	0.6	20	80
10.00	0.6	60	40
13.00	0.6	60	40

Preparation of solutions:

Preparation of standard solution

A stock solution of Bosentan (1.0 mg·mL–1) was prepared by dissolving appropriate amount in the diluent. Working solutions were prepared from above stock solution for assay and related substances and stock solution of impurities (mixture of imp-1, imp-2 imp-3 imp-4 and imp-5) at a concentration of 10 µg·mL–1 was also prepared in diluent.

Preparation of Test solutions (Assay):

Bosentas tablets contain 62.5 mg of Bosantan .Twenty tablets (62.5 mg) were weighed and the average weight was calculated. The tablets were powdered in a mortar and a sample of the powder equivalent to 50 mg of the active pharmaceutical ingredient (Bosentan) was transferred to 50 mL volumetric flask. Approximately 40 mL diluent was added and the flask was placed on rotatory shaker for 10 min and sonicated for 30 min to dissolve the material completely. The solution was then diluted to 50 mL and centrifuged at 3000 rpm for 10 min. The supernatant was collected and filtered through a 0.45 µm pore size Syringe filter. The filtrate was used as sample solution for impurities (1000 ppm), the above filtered solution on dilution of 5 mL to 50 mL (100 ppm) used as test solution for assay.

Quantification:

Equal volumes, (2µL), of the standard preparations and the test preparations that contain

Bosentan and its related substances were injected into the chromatograph and the chromatograms were recorded. The responses (peak area) for the major peaks were measured and the quantity of Bosentan or related substance was calculated from the equation Cs (Au / As) where Au and As are the areas under the corresponding peaks and Cs is the concentration of Bosentan and its related substance in the standard solution.

Method validation

The method validation was performed as per ICH Guidelines[12].

Linearity, Limit of detection, Limit of quantification

The degree of linearity was assessed by the correlation coefficient, y-intercept, and slope. The limit of detection, LOD and the limit of quantitation LOQ have been estimated for related substances as 3 S.D. and 10 S.D. of the y intercept and slope.

Precision

The precision was performed by preparing six individual preparations as per the method of analysis and evaluated for percentage of Bosentan and its percentage of individual and total impurities.

Accuracy

The samples were prepared by spiking the Bosentan and its impurities stock solutions into the Placebo mixture and the percent recovery was estimated.

Solution stability

The solutions prepared was tested at initial, 24hrs and 48Hrs by maintaining at room temperature and estimated for Bosentan and its impurity content.

Robustness

Robustness was conducted by making the variations in flow rate, Column oven temperature.

Ruggedness

The prepared solutions were filtered through 0.45 µ PVDF syringe filter and 0.45 µ PVDF syringe filter and evaluated against the centrifuged sample.

Intermediate precision

The test was performed with another analyst on different day, different system and different column and the impurity contents were reported.

Forced degradation studies

The forced degradation studies conditions and % degradation s mentioned in the results (Table: 9) section.

Study for Uneluted peaks:

Since the runtimes are lower, a study conducted on all the stressed samples for knowing the retained peaks by increasing the acetonitrile to 90% till 15 minutes.

Equivalency with the API Vendor HPLC method:

The developed UPLC method was tested for equivalency with API Vendor method in three steps.

System suitability equivalence:

The System suitability parameters in the API Vendor method and develop method are compared with the obtained values.

API Analysis equivalence:

The results obtained with the Same API batch analysis with the API Vendor method and the developed method, results were discussed.

Reference Product analysis equivalence:

The results obtained with the Same Bosantas tablet batch analysis with the API Vendor method and the developed method, results were discussed.

RESULTS AND DISCUSSIONS

The impurity mix with the resolution of more than 3.0, Blank, Placebo chromatograms without interference was obtained after finalization of method was as below

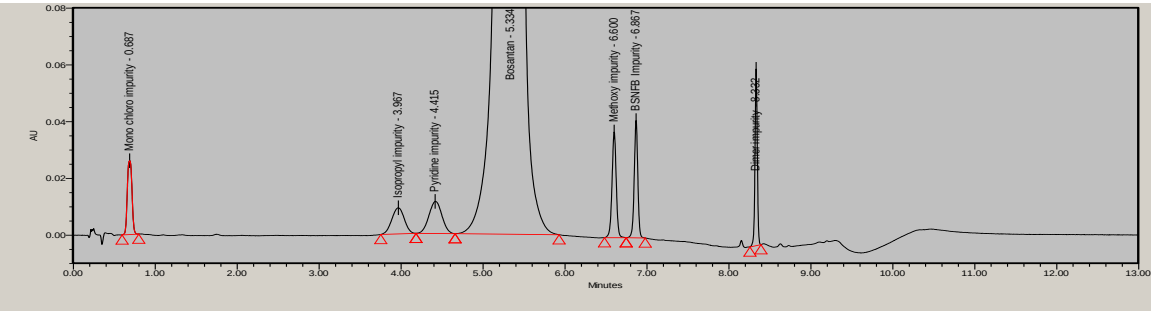

Figure 2: Impurities spiked sample chromatogram.

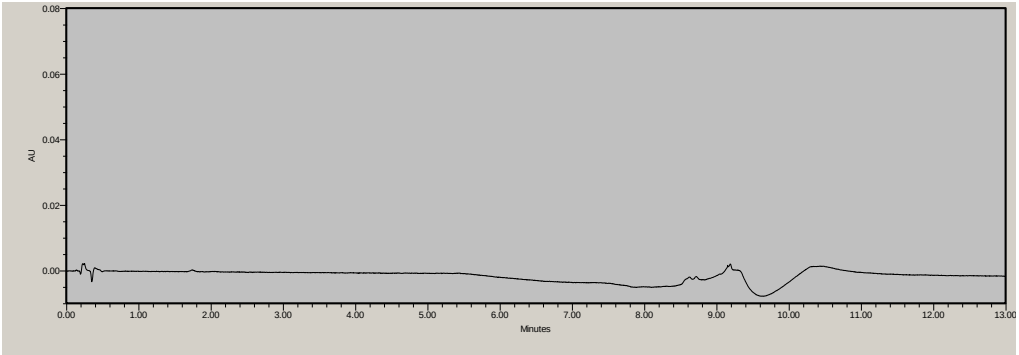

Figure 3: Blank chromatogram.

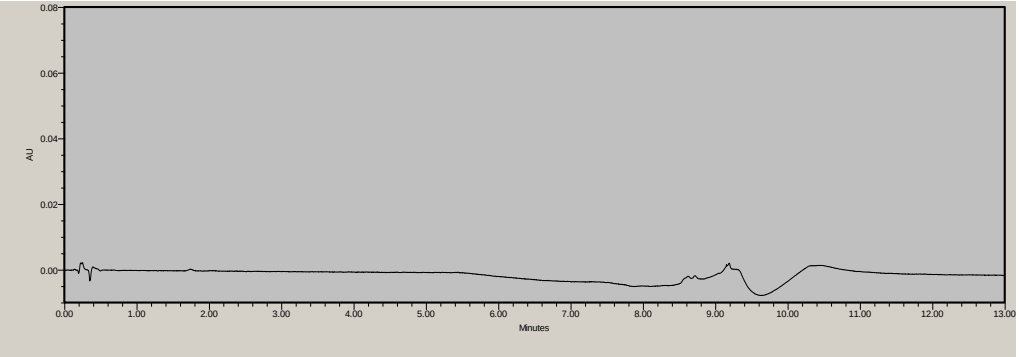

Figure 4: Placebo chromatogram.

Assay method validation results:

Specificity:

Blank interference:

The diluent was injected as a blank; it was found that there was no interference observed in placebo preparation with the Bosentan peak.

Placebo interference:

With the equivalent weight of sample the placebo preparation was prepared and injected into the system and interference checked, it was found that there was no interference observed in placebo preparation with the Bosentan peak.

Impurity interference:

Impurity solution was prepared at 5 % level of test concentration and injected into the system and checked for the interference, it was found that impurities are not interfering with the Bosentan peak.

Linearity:

The linearity was performed at 6 levels of the targeted 100 ppm (25%, 50%, 80%, 100%, 200%, 300% levels) and the area results are plotted against the concentration, the correlation coefficient observed was 0.9999.

Precision:

By following the procedure in 2.5.2 section, six sample preparations are prepared and calculated the assay values and the Percent relative standard deviation was 0.34 shows that the method was precise as per the ICH limits.

Accuracy:

The accuracy was performed in triplicate by spiking the Bosentan into the placebo mixture at 50%, 100%, 150%, 200% and 300% of test concentration; from the area recovery values are calculated. The average recovery values are obtained within 99 to 101% shows that the method

was accurate as per the ICH limits.

Solution stability:

The first three solutions prepared in the 2.5.2 section were checked for Bosentan content at 24 and 48 hours by keeping it in closed container at room temperature, the variation from the initial value was 0.36% at 24 Hours and 0.41% at 48 hours, the results are within 0.5% shows the solution was stable for 48 hours.

Robustness:

To check the effect of deliberate changes in the method, the variation inflow rate (±0.05 mL) and variation in temperature (± 5ºC) are studied; result shows no effect on the method.

Ruggedness:

From the stock solutions of 3.1.3 the solution are prepared by filtering through PVDF PTFE 0.45 µm filter papers and the content of bosentan was tested. The results obtained are within 0.5%.

Intermediate precision:

Assay was performed by another analyst on different day, different system, and different column; the variation between the two analysts was less that 0.3 %, it shows that method was reproducible.

Table 2: Validation results of Bosentan in assay method

Parameter	Results	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 25% to 300% (R^2 value=0.9999)	
Precision:	% RSD of impurity for six preparations= 0.34	
Accuracy	**%Level**	**%Recovery**
	50%	99.7
	100%	100.1
	150%	100.3
	200%	100.0
	300%	99.8
Solution stability	1)% Difference at 24 Hrs=0.36% 2)%Difference at 48 Hrs=0.41%	
Robustness	Flow rate variation-System suitability passes Temperature variation system suitability passes	
Ruggedness	Filter validation:Variation between PVDF &PTFE 0.45 micron filters=0.27%	
Intermediate precision	% Assay variation=0.23%	

Impurity method validation results:

Specificity:

Blank interference:

The diluent was injected as a blank; it was found that there was no interference observed in

placebo preparation with the Impurity peaks.

Placebo interference:

With the equivalent weight of sample the placebo preparation was prepared and injected into the system and interference checked, it was found that there was no interference observed in placebo preparation with the Impurity peaks.

Impurity interference:

Impurity solution was prepared at 5 % level with the Bosentan API at test concentration and injected into the system and checked for the interference, it was found all the impurities are separated with minimum resolution of 3.0, indicating no impurity interference.

Linearity:

The linearity was performed at 8 levels of the targeted diluted standard concentration 10 ppm (10%, 20%, 40%, 50%, 100%, 200% and 400% levels) and the area results are plotted against the concentration, the correlation coefficient observed was above 0.998 shows that the method was linear.

Precision:

By following the procedure in 2.5.3 section, six sample preparations are prepared and calculated the impurity content values and the Percent relative standard deviation for four impurities was below 2% shows that the method was precise.

Accuracy:

The accuracy was performed in triplicate by spiking the impurity stock solutions into the placebo mixture at 10%, 20%, 50%, 80% 100% and 200% of diluted standard concentration; from the obtained area recovery values are calculated. The average recovery values are obtained within 85 to 115% shows that the method was accurate as per the ICH limits.

Solution stability:

The first three solutions prepared in the 3.2.3 section were checked for individual and total impurities contents at 24 and 48 hours by keeping it in closed container at room temperature, the variation from the initial individual impurity not more than 0.04 %and total impurity content value was below 0.1% shows the solution was stable for 48 hours.

Robustness:

To check the effect of deliberate changes in the method, the variation inflow rate (±0.05 mL) and variation in temperature (± 5ºC) are studied; result shows no effect on system suitability and resolution 1.5 was maintained in all the changes.

Ruggedness:

From the stock solutions of 3.2.3 the solution are prepared by filtering through PVDF PTFE 0.45 µm filter papers and the content of individual impurities and total impurities content for both filters was tested. The results obtained are within 0.02%.

Intermediate precision:

Impurity test was performed by another analyst on different day, different system, and different column; the variation between the two analysts was less that 0.07%, it shows that method was reproducible.

The validation results obtained with the related compounds are summarized in below (Table 3, 4, 5, 6, 7 and 8)

Table 3: Validation results of mono chloro impurity in RS method

Parameter	Results	
RRT	0.13	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 0.84 ppm to 21 ppm (R^2 value=0.999)	
LOD and LOQ	LOD=0.41 ppm and LOQ=1.24 ppm	
Precision:	% RSD of impurity for six preparations= 0.83	
Accuracy	**%Level**	**%Recovery**
	10%	104.4
	20%	102.6
	50%	93.8
	80%	94.9
	100%	95.6
	200%	93.5
Solution stability	1)% Difference at 24 Hrs=0.01%	
	2)%Difference at 48 Hrs=0.01%	
Robustness	Flow rate variation-System suitability passes	
	Temperature variation system suitability passes	
Ruggedness	Filter validation: Variation between PVDF &PTFE 0.45 micron filters=0.00%	
Intermediate precision	Individual impurity variation=0.02% Total impurity variation=0.06%	

Table 4: Validation results of Isopropyl impurity in RS method

Parameter	Results	
RRT	0.75	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 0.84 ppm to 21 ppm (R^2 value=0.999)	
LOD and LOQ	LOD=0.07 ppm and LOQ=0.21 ppm	
Precision:	% RSD of impurity for six preparations=1.57	
Accuracy	**%Level**	**%Recovery**
	10%	103.5
	20%	103.9
	50%	98.2

	80%	97.8
	100%	95.1
	200%	96.9
Solution stability	1)% Difference at 24 Hrs=0.02%	
	2)%Difference at 48 Hrs=0.06%	
Robustness	Flow rate variation-System suitability passes	
	Temperature variation system suitability passes	
Ruggedness	Filter validation: Variation between PVDF &PTFE 0.45 micron filters=0.00%	
Intermediate precision	Individual impurity variation=0.04% Total impurity variation=0.06%	

Table 5: Validation results of pyrimidinone impurity in RS method

Parameter	**Results**	
RRT	0.83	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 0.8 ppm to 21 ppm (R^2 value=0.999)	
LOD and LOQ	LOD=1.23 ppm and LOQ=3.73 ppm	
Precision:	% RSD of impurity for six preparations=1.35	
Accuracy	**%Level**	**%Recovery**
	10%	104.4
	20%	103.4
	50%	97.1
	80%	96.2
	100%	95.0
	200%	91.7
Solution stability	1)% Difference at 24 Hrs=0.01%	
	2)%Difference at 48 Hrs=0.01%	
Robustness	Flow rate variation-System suitability passes	
	Temperature variation system suitability passes	
Ruggedness	Filter validation: Variation between PVDF &PTFE 0.45 micron filters=0.00%	
Intermediate precision	Individual impurity variation=0.04% Total impurity variation=0.06%	

Table 6: Validation results of Methoxy impurity in RS method

Parameter	**Results**	
RRT	1.24	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 0.8 ppm to 21 ppm (R^2 value=0.999)	
LOD and LOQ	LOD=0.03 ppm and LOQ=0.10 ppm	
Precision:	% RSD of impurity for six preparations=1.37	
Accuracy	**%Level**	**%Recovery**
	10%	95.4
	20%	98.1
	50%	95.4
	80%	94.6
	100%	94.6
	200%	93.3
Solution stability	1)% Difference at 24 Hrs=0.02%	

	2)%Difference at 48 Hrs=0.02%
Robustness	Flow rate variation-System suitability passes
	Temperature variation system suitability passes
Ruggedness	Filter validation:
	Variation between PVDF &PTFE 0.45 micron filters=0.01%
Intermediate precision	Individual impurity variation=0.03% Total impurity variation=0.06%

Table 7: Validation results of BSN4FB impurity in RS method

Parameter	**Results**	
RRT	1.29	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 0.8 ppm to 21 ppm (R^2 value=1)	
LOD and LOQ	LOD=0.29 ppm and LOQ=0.87 ppm	
Precision:	% RSD of impurity for six preparations=0.45	
Accuracy	**%Level**	**%Recovery**
	10%	105.6
	20%	98.0
	50%	94.8
	80%	94.5
	100%	94.2
	200%	92.3
Solution stability	1)% Difference at 24 Hrs=0.01%	
	2)%Difference at 48 Hrs=0.06%	
Robustness	Flow rate variation-System suitability passes	
	Temperature variation system suitability passes	
Ruggedness	Filter validation:	
	Variation between PVDF &PTFE 0.45 micron filters=0.01%	
Intermediate precision	Individual impurity variation=0.04%	
	Total impurity variation=0.06%	

Table 8: Validation results of Dimer impurity in RS method

Parameter	**Results**	
RRT	1.57	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 0.8 ppm to 21 ppm (R^2 value=0.999)	
LOD and LOQ	LOD=2.68 ppm and LOQ=8.11 ppm	
Precision:	% RSD of impurity for six preparations=0.78	
Accuracy	**%Level**	**%Recovery**
	10%	97.9
	20%	98.4
	50%	102.2
	80%	96.8
	100%	94.9
	200%	90.0
Solution stability	1)% Difference at 24 Hrs=0.00%	
	2)%Difference at 48 Hrs=0.00%	
Robustness	Flow rate variation-System suitability passes	
	Temperature variation system suitability passes	

Ruggedness	Filter validation: Variation between PVDF &PTFE 0.45 micron filters=0.00%
Intermediate precision	Individual impurity variation=0.04% Total impurity variation=0.06%

Study of un eluted peak:

The study shows that no peak eluted, proved that there was no un eluted peak with the developed method.

Forced degradation studies:

To prove the stability indicating power of the method the forced degradation studies are carried out. The degradation reagents were (30 mL) added after the disintegration kept on reflux for the specified time. In each condition the individual % of impurities and total impurities and assay are calculated. The mass balance obtained from the experiment was ranged 99 to 100%. In all the forced degradation conditions peak purity of Bosentan and major degradent peaks are passed, it shows that the developed method was stability indicating.

Acid degradation:

It was performed with IN Hydrochloric acid for 24 hours and the degradation observed was 0.74% and the assay value was 99.2,In Impurities test the peak purity of Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in acidic condition.

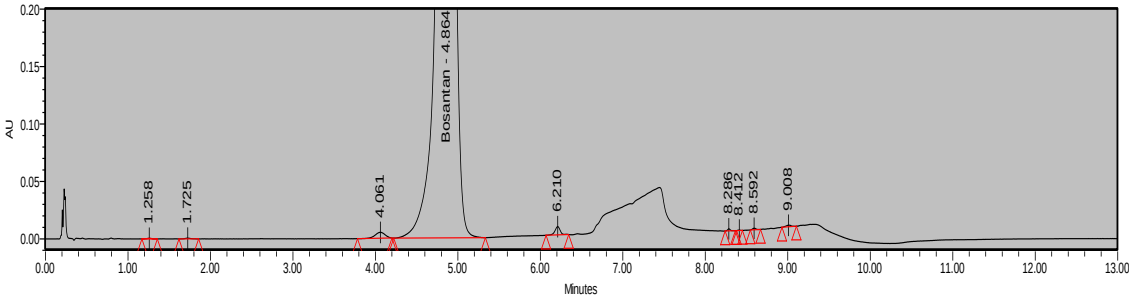

Figure 5: Acid degraded sample chromatogram

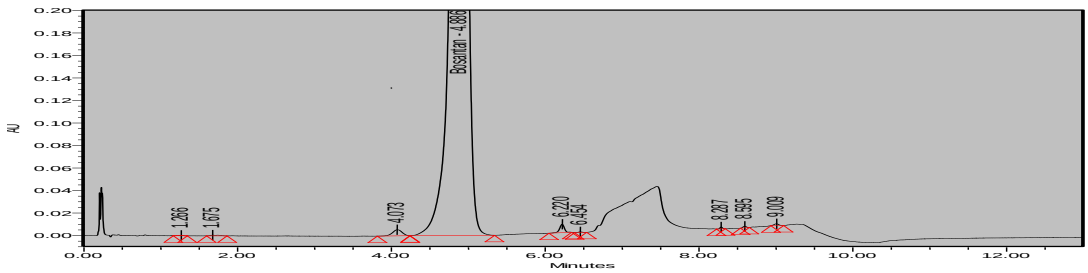

Figure 6: Base stressed sample chromatogram

Base degradation:

It was performed with IN sodium hydroxide for 24 hours and the degradation observed was

0.80% and the assay value was 99.1%,In Impurities test the peak purity of Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in Base degradation.

Peroxide degradation:

It was performed with 10% Hydrogen peroxide for 24 hours and the degradation observed was 0.66% and the assay value was 99.3%,In Impurities test the peak purity of Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in Peroxide degradation.

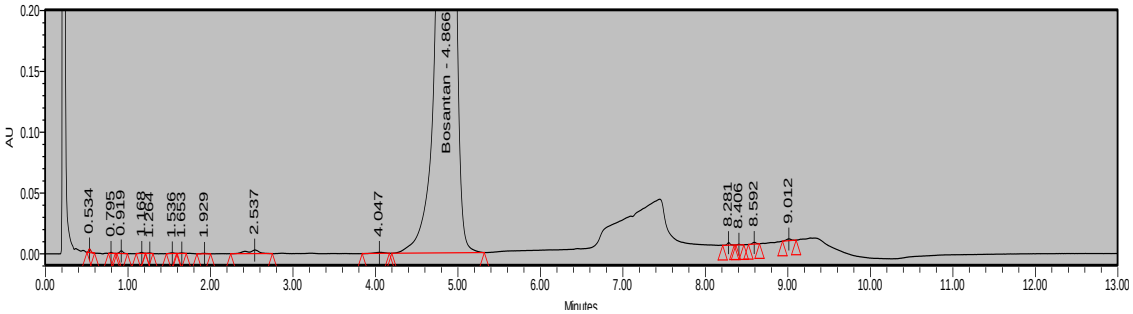

Figure 7: Peroxide stressed sample chromatogram

Water degradation:

It was performed with Milli Q water for 24 hours and the degradation observed was 0.18% and the assay value was 99.8%,In Impurities test the peak purity of Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in Water degradation.

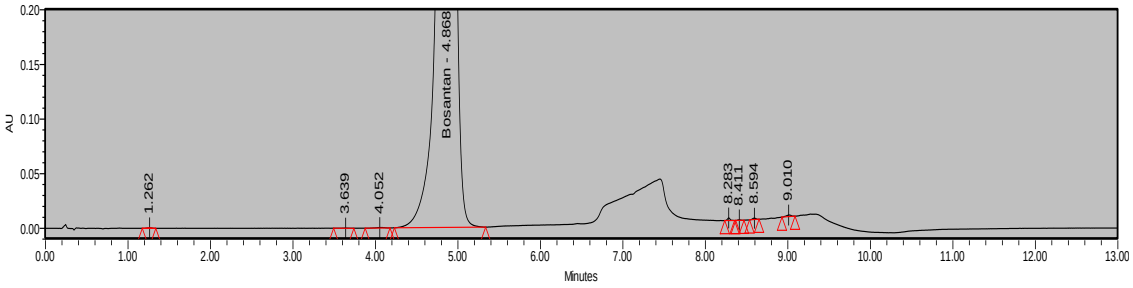

Figure 8: Water stressed sample chromatogram

Thermal degradation:

It was performed at 50ºC water for 48 hours and the degradation observed was 0.18% and the assay value was 99.8%,In Impurities test the peak purity of Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in Thermal degradation.

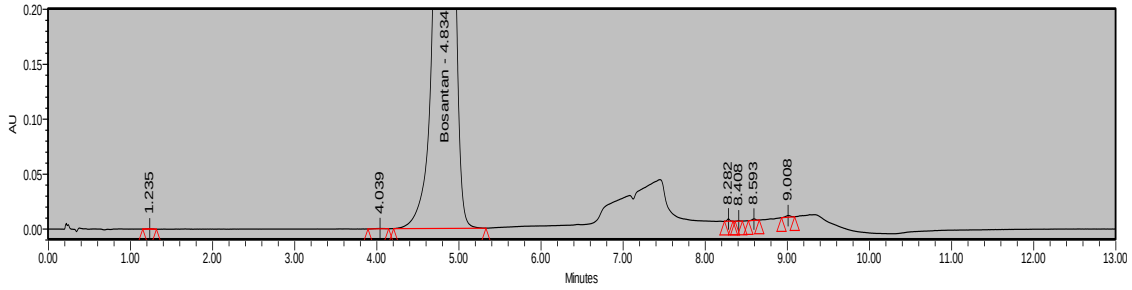

Figure 9: Thermal stressed sample chromatogram

Photo degradation:

It was performed till 1.2 million Lux hours visible light and 200 Watts UV exposure, the degradation observed was 0.18% and the assay value was 99.8%,In Impurities test the peak purity of Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in Photo degradation.

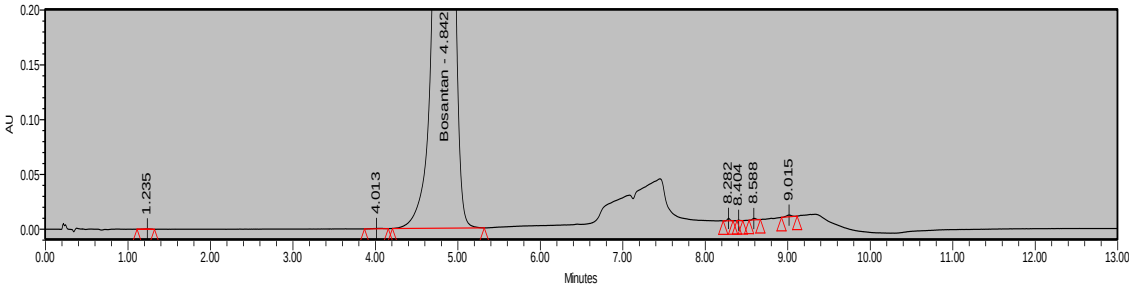

Figure 10: Light stressed sample chromatogram.

The overall summery of forced degradation results was as below.

Table 9: Forced degradation study results compilation

Type	Condition &Duration	% Degradation	%Assay	Peak purity
Acid	1N Hcl,24 hours,50°C	0.74	99.2	Passes
Base	1N NaoH,1 day,50°C	0.80	99.1	Passes
Peroxide	10% H_2O_2,1 day,50°C	0.66	99.3	Passes
Water	Water,1 day,50°C	0.18	99.8	Passes
Thermal	1 days,50°C	0.18	99.8	Passes
Photo	1.2 million Lux hours	0.18	99.8	Passes

System suitability equivalence:

The difference in the results between developed method and API Vendor method was much lesser. The critical pair resolution was maintained above 2.0 in the developed method, it shows that the results are comparable to that of API Vendor method results.

Table 10: System suitability equivalence table

Parameter	API Vendor method	UPLC Method
Assay Standard %RSD	Not more than 2%	Not more than 2%
Critical pair resolution (Between Impurity-2 imp &Impurity-3)	2.4	4.1

API Batch analysis results equivalence:

The results obtained with the developed method was compared with the API Vendor method results, the variation in assay and impurities results was below 0.1%, proves that the method was equivalent to the API Vendor method with 10 minutes runtime.

Table 11: API Analysis results equivalence table

Details	API Vendor method Results	UPLC Method results
B.No:BNm0060311		
Known impurity	0.06%	0.06%
Any unknown individual impurity	0.01%	0.03%
Total impurity	0.15%	0.17%
Assay	99.7%	99.8%

Reference product analysis results equivalence:

The results obtained with the developed method shows the difference less than 0.05% with API Vendor method, shows that the developed method is equivalent to that of API Vendor method with 10 minutes Runtime.

Table 12: Reference product analysis (Bosantas-cipla) equivalence table

Details	API Vendor method Results	UPLC Method results
B.No:2345AF		
1)Known impurity	1)0.01%	1)0.01%
2)Any unknown individual impurity	2)0.04%	2)0.04%
3)Total impurity	3)0.22%	3)0.24%
4) Assay	4)100.1%	4)100.0%

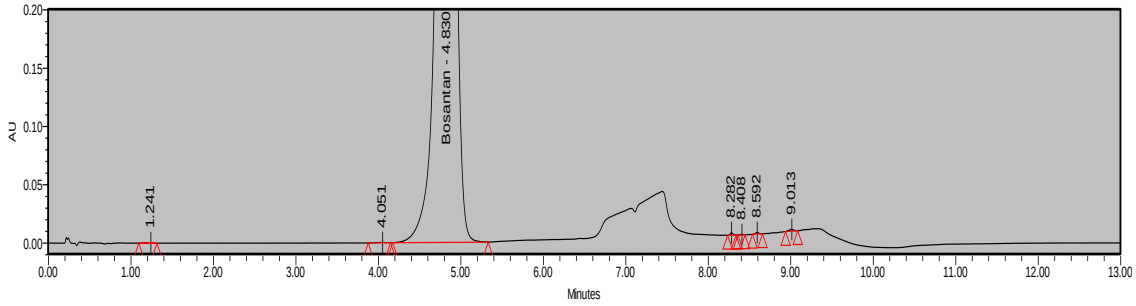

Figure 11: Bosentan sample chromatogram

CONCLUSION:

An UPLC method for related compounds in the commercial drug products and in the tablet formulation was validated in this study. Bosentan, Bosentan degradants and impurities gave chromatograms of very well resolved peaks which indicate the specificity of the method and the possibility of using it as an indicator of stability. Slight changes in the experimental conditions did not affect significantly the resolution of the compounds of interest or their percent recoveries indicating the robustness of the method. All the statistical values (percent recovery, RSD, %, the

slope and the intercept, LOD and LOQ) calculated were within the acceptable limits and shown equivalent to the API Vendor method. The method can be used for estimation of Bosentan and its related impurities in bulk drugs and its tablet dosage forms for quality control purposes.

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