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A stability indicating RP-UPLC method for simultaneous estimation of Sildenafil citrate, Bosentan and their 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 Sildenafil citrate, 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 0.4 mL/min and UV detector set at 220 nm has shown a good chromatographic separation for sildenafil citrate, Bosentan and its related substances. The developed method was validated as per ICH Guidelines. The developed UPLC method has run time of only 20 minutes making the method productive and may be applied for Quality control Testing.

Keywords: sildenafil citrate, bosentan, Stability indicating, RP-UPLC, Equivalency.

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INTRODUCTION

Sildenafil citrate[1] is a therapy used for erectile dysfunction by acting as a selective inhibitor of cyclic guanosine mono phosphate(cGMP)-specific phosphor diesterase type 5(PDES).Sildenafil is chemically known as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1 H pyrazolo[4,3-d]pyrimidine-5-yl)-4-ethoxy phenyl]sulfonyl]-4-methyl piperizine citrate has the following structural formulae$C_{28}H_{38}N_6O_{11}S$. And its molecular mass: base: 474.6 g mol−1; citrate: 666.7 g mol−1. Sildenafil enhances relaxation of the corpus cavernosal smooth muscle, which in turn increases blood flow into the cavernosal spaces, thus leading to increased intra cavernosal pressure, a key factor in producing an erect penis[2-3].

Sildenafil citrate, sold under the names Viagra, was a drug used to treat male erectile dysfunction (impotence) and pulmonary arterial hypertension (PAH). However, the introduction of sildenafil resulted to its widespread use as well as its abuse. Therefore, specific, accurate, and robust determination of this drug is widely required. Several methods have been developed for this purpose. Pistos et al[4] have proposed a HPLC method for determination of sildenafil and its active metabolite (N-desmethyl sildenafil) in human blood. Determination of sildenafil citrate in human plasma [5-9] and in pharmaceutical formulations[10-15] using chromatographic methods has been reported.

Extensive literature survey revealed that, Ultra performance liquid chromatography method was not reported for the sildenafil and its related substances in its Bulk drugs and its formulations. Reports have been appeared describing accurate analytical and spectrophotometric techniques for quantification and assay of sildenafil. For related substances [16-38] was reported most of these methods are time consuming, expensive, suffer from lack of selectivity and require careful control of conditions and considerable time for routine control analysis.

The HPLC method of analysis as reported in the pharmacopoeia is adequate to separate all the process related as well as the probable degradation related substances in both the API as well as finished dosage form. However, this method suffers from an inordinately long run time (60>) and hence is inherently expensive.

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 [39–46]. Further Tracleer has been demonstrated to be effective in remodelling the pulmonary vascular tree through several mechanisms including vasodilatation, antifibrotic and antithrombotic actions [47]. 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 [48-50].

The reported HPLC method [51] 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.

The combination of sildenafil and bosentan was approved by FDA and detailed literature was available [52-53].

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 assay and related substances method for accurate quantification of four impurities of sildenafil citrate and six potential process impurities in bosentan monohydrate samples as per International Conference on Harmonization (ICH) recommendations. Intensive stress studies are carried out on sildenafil citrate and 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[54-55]. 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 [56-57].

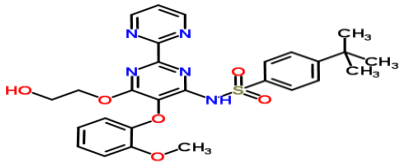

Figure1: Bosentan chemical structure

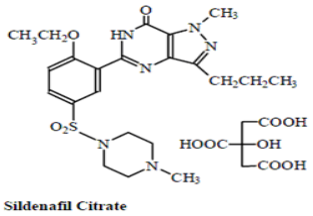

Figure2: Sildenafil citrate chemical structure

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 AR Grade was from Merck. Sildenafil citrate and Bosentan and its impurities were obtained from MSN Laboratories. Viagra tablets are obtained from Pfizer. Bosantas tablets were obtained from cipla Ltd, India.

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

1) Sildenafil amino impurity: 4-amino-1-methyl-3-propyl-1H-pyrazole-5-carboxamide.

2) Sildenafil adduct impurity: 4-ethoxy-3-(1-methyl-7-oxo-3-propyl-6, 7, dihydro-1H-pyrazolo [4, 3-d] pyrimidin-5-yl) benzene-1-sulfonyl chloride.

3) Sildenafil coupled impurity: 4-(2-ethoxy benzamido-1-methyl-3-propyl-1H-pyrazole-5-carboxamide..

4) Sildenafil cyclised impurity: 5-(2-ethoxy phenyl)-1-methyl-3-propyl-1H-pyrazino [4, 3,-d] pyramidin-7(6H)-one.

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

6) Bosentan 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.

7) Bosentan 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.

8) Bosentan 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.

9) Bosentan 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.

10) Bosentan 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 0.2M KH_2PO_4 and 2 mL of tri ethyl amine pH adjusted to 2.0 with phosphoric acid: methanol and acetonitrile (50:50) gradient at flow rate 0.4 mL·min–1. The sildenafil amino impurity was coeluting with the blank peak and sildenafil coupled impurity was co eluting with the sildenafil main peak.

Different columns such as BEH C18, BEH C8 and different buffers such as, 0.2 M Ammonium acetate, formic acid, trifluoro acetic acid were also tried with different isocratic and gradient methods to achieve the chromatographic separation. But long retention times and less resolution were still unavoidable.

With 0.1% trifluoroacetic acid, Sildenafil coupled impurity and sildenafil 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, Bosantan Iso propyl impurity and pyrimidinone impurity are co eluting with bosentan peak. 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 .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 two drugs, its degradation products and ten 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 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 0.4 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 1µL. 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
0.0	0.4	85	15
5.0	0.4	70	30
7.0	0.4	65	35
10.0	0.4	60	40
15.0	0.4	55	45
17.0	0.4	10	90
18.0	0.4	85	15
20.0	0.4	85	15

Preparation of solutions:

Preparation of standard solution

A stock solution of sildenafil citrate and Bosentan (1.0 mg·mL–1) was prepared differently 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 to imp-10) at a concentration of 10 µg·mL–1 was also prepared in diluent.

Preparation of Test solutions (Sildenafil):

Twenty tablets were powdered and accurately weighed portions equivalent to 100 mg sildenafil Citrate were transferred to100 ml volumetric flasks. Disintegrated with 20 mL of water and Acetonitrile was added and the solutions were sonicated and centrifuged as above and the supernatant was used as Related substances test solution with 1.0 mg sildenafil citrate per ml, the filtered solution diluting 5 mL to 50 mL used for Assay.

Preparation of Test solutions (Bosantan):

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, (1µL), 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[56-57].

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 Sildenafil Citate, Bosentan and its percentage of individual and total impurities.

Accuracy

The samples were prepared by spiking the Sildenafil citrate, 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 sildenafil citrate, Bosentan and its individual and total impurity content.

Robustness

Robustness was conducted by making the variations in flow rate, Column oven temperature and checked for the area standard deviation and retention times of analytes.

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 API contents and the impurity contents were reported.

Forced degradation studies

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

Equivalency with the Available pharmacopeia method:

The developed UPLC method was tested for equivalency with Available pharmacopeia method in three steps.

System suitability equivalence:

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

API Analysis equivalence:

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

Reference Product analysis equivalence:

The results obtained with the single Viagra and bosantas tablet batch analysis (Individually) with the Pharmacopeia method and the developed method, results were discussed.

RESULTS AND DISCUSSIONS

The impurity mix with the resolution of more than 2.0, Blank, Placebo chromatograms without interference was obtained after finalization of method was as below

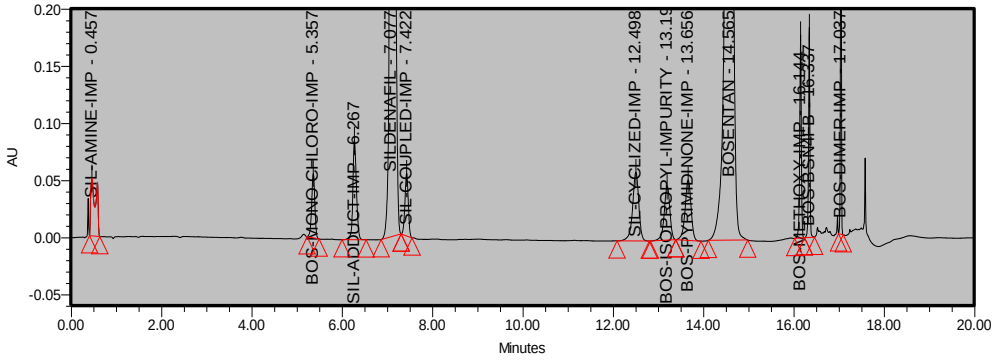

Figure 3: Impurities spiked sample chromatogram

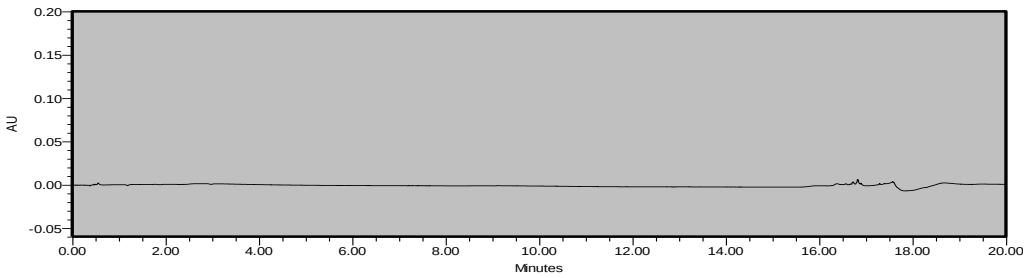

Figure 4: Blank chromatogram.

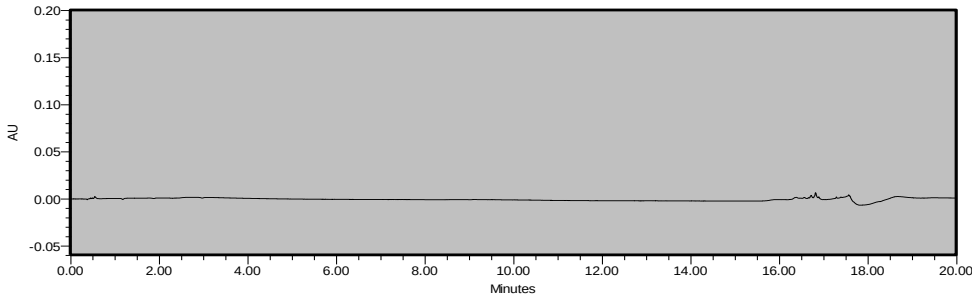

Figure 5: Placebo chromatogram.

Assay method validation results:

Specificity:

Blank interference:

The diluent was injected as a blank; it was found that there was no interference observed in Blank preparation with the sildenafil and 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 sildenafil and 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 sildenafil and 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 above 0.999.

Precision:

By following the procedure in 2.7.2 section, six sample preparations are prepared and calculated the assay values and the Percent relative standard deviation was 0.44 for sildenafil and 0.26 for

bosentan shows that the method was precise as per the ICH limits.

Accuracy:

The accuracy was performed in triplicate by spiking the sildenafil and bosentan into its individual 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 2.7.2 section were checked for sildenafil and Bosentan content at 24 and 48 hours by keeping it in closed container at room temperature, the variation from the initial value was 0.22% at 24 Hours and 0.31% at 48 hours for sildenafil and0.31% at 24 Hours and 0.42% at 48 hours for bosentan 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 and PTFE 0.45 µm filter papers and the content of sildenafil and bosentan was tested. The results obtained are within 0.5%.

Table 2: Validation results of Sildenafil 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= 0.44
Accuracy	**%Level** **%Recovery** 50% 99.3 100% 100.2 150% 100.1 200% 100.0 300% 99.7
Solution stability	1)% Difference at 24 Hrs=0.22% 2)%Difference at 48 Hrs=0.31%
Robustness	Flow rate variation-System suitability passes Temperature variation system suitability passes
Ruggedness	Filter validation: 1)Variation between centrifuged sample PVDF 0.45 micron filters=0.2% 2)Variation between centrifuged sample PTFE 0.45 micron filters=0.1%
Intermediate precision	% Assay variation=0.4%

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 0.4 % for sildenafil and 0.54 % for bosentan, it shows that method was reproducible.

Table 3: Validation results of Bosantan 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= 0.26
Accuracy	**%Level** **%Recovery**
	50% 99.7
	100% 100.0
	150% 99.9
	200% 99.8
	300% 100.1
Solution stability	1)% Difference at 24 Hrs=0.31%
	2)%Difference at 48 Hrs=0.42%
Robustness	Flow rate variation-System suitability passes
	Temperature variation system suitability passes
Ruggedness	Filter validation:
	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.1%
	2)Variation between centrifuged sample PTFE 0.45 micron filters=0.1%
Intermediate precision	% Assay variation=0.5%

Impurity method validation results:

Specificity:

Blank interference:

The diluent was injected as a blank; it was found that there was no interference observed in blank preparation with the sildenafil and bosentan 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 sildenafil and bosentan 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 2.0, indicating no impurity interference.

Linearity:

The linearity was performed at 7 levels of the targeted diluted standard concentration 10 ppm (10%, 20%, 40%, 50%, 100% and 400% levels) and the area results are plotted against the

concentration, the correlation coefficient observed was above 0.99 shows that the method was linear.

Precision:

By following the procedure in 2.7.2 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 400% 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 and PTFE 0.45 µm syringe filter and the content of individual impurities and total impurities was compared with centrifuged sample. The results obtained are within 0.1%.

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 than 0.1%, it shows that method was reproducible. The validation results obtained with the related compounds are summarized in below (Table 4, 5, 6, 7, 8, 9,10,11,12, and 13)

Table 4: Validation results of Sildenafil amino impurity in RS method

Parameter	Results
RRT	0.1 with respect to sildenafil
Specificity	Blank interference, Placebo interference, Impurity interference was nil.
Linearity	Established from 1 ppm to 42 ppm (R^2 value=1)

LOD and LOQ	LOD=2.16 ppm and LOQ=4.56 ppm	
Precision:	% RSD of impurity for six preparations= 0.78	
Accuracy	**%Level**	**%Recovery**
	10%	105.5
	20%	112.4
	40%	105.3
	50%	105.3
	100%	102.0
	400%	98.9
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	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.01%	
	2)Variation between centrifuged sample PTFE 0.45 micron filters=0.01%	
Intermediate precision	Individual impurity variation=0.04% Total impurity variation=0.06%	

Table 5: Validation results of Sildenafil adduct impurity in RS method

Parameter	**Results**	
RRT	0.86 with respect to sildenafil	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 1 ppm to 42 ppm (R^2 value=0.999)	
LOD and LOQ	LOD=0.63 ppm and LOQ=1.92 ppm	
Precision:	% RSD of impurity for six preparations= 0.76	
Accuracy	**%Level**	**%Recovery**
	10%	96.4
	20%	101.5
	40%	101.4
	50%	100.5
	100%	101.5
	400%	102.7
Solution stability	1)% Difference at 24 Hrs=0.03%	
	2)%Difference at 48 Hrs=0.03%	
Robustness	Flow rate variation-System suitability passes	
	Temperature variation system suitability passes	
Ruggedness	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.00%	
	2)Variation between centrifuged sample PTFE 0.45 micron filters=0.00%	
Intermediate precision	Individual impurity variation=0.03% Total impurity variation=0.06%	

Table 6: Validation results of Sildenafil coupled impurity in RS method

Parameter	**Results**	
RRT	1.02 with respect to sildenafil	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 1 ppm to 42 ppm (R^2 value=0.999)	
LOD and LOQ	LOD=2.10 ppm and LOQ=6.35 ppm	
Precision:	% RSD of impurity for six preparations= 1.20%	
Accuracy	**%Level**	**%Recovery**
	10%	93.5
	20%	87.6

	40%	94.7
	50%	97.8
	100%	106.5
	400%	101.6
Solution stability	1)% Difference at 24 Hrs=0.03%	
	2)%Difference at 48 Hrs=0.03%	
Robustness	Flow rate variation-System suitability passes	
	Temperature variation system suitability passes	
Ruggedness	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.01%	
	2)Variation between centrifuged sample PTFE 0.45 micron filters=0.01%	
Intermediate precision	Individual impurity variation=0.02% Total impurity variation=0.06%	

Table 7: Validation results of Sildenafil cyclised impurity in RS method

Parameter	**Results**	
RRT	1.71 with respect to sildenafil	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 1 ppm to 42 ppm (R^2 value=0.999)	
LOD and LOQ	LOD=0.64 ppm and LOQ=1.93 ppm	
Precision:	% RSD of impurity for six preparations= 1.07%	
Accuracy	**%Level**	**%Recovery**
	10%	97.8
	20%	97.9
	40%	99.8
	50%	100.7
	100%	99.9
	400%	101.5
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	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.00%	
	2)Variation between centrifuged sample PTFE 0.45 micron filters=0.00%	
Intermediate precision	Individual impurity variation=0.03% Total impurity variation=0.06%	

Table 8: Validation results of Bosantan mono chloro impurity in RS method

Parameter	**Results**	
RRT	0.37 with respect to bosentan	
Specificity	Blank interference, Placebo interference, Impurity interference was nil.	
Linearity	Established from 1 ppm to 42 ppm (R^2 value=0.999)	
LOD and LOQ	LOD=0.34 ppm and LOQ=1.03 ppm	
Precision:	% RSD of impurity for six preparations= 1.01%	
Accuracy	**%Level**	**%Recovery**
	10%	104.3
	20%	100.4
	40%	98.0
	50%	97.4
	100%	94.2
	400%	94.7
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	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.00%
	2)Variation between centrifuged sample PTFE 0.45 micron filters=0.00%
Intermediate precision	Individual impurity variation=0.04% Total impurity variation=0.08%

Table 9: Validation results of Bosantan isopropyl impurity in RS method

Parameter	**Results**
RRT	0.90 with respect to bosentan
Specificity	Blank interference, Placebo interference, Impurity interference was nil.
Linearity	Established from 1 ppm to 42 ppm (R^2 value=0.999)
LOD and LOQ	LOD=0.08 ppm and LOQ=0.26 ppm
Precision:	% RSD of impurity for six preparations= 0.67%
Accuracy	**%Level** **%Recovery**
	10% 101.8
	20% 96.8
	40% 94.6
	50% 94.5
	100% 93.6
	400% 93.7
Solution stability	1)% Difference at 24 Hrs=0.04%
	2)%Difference at 48 Hrs=0.04%
Robustness	Flow rate variation-System suitability passes
	Temperature variation system suitability passes
Ruggedness	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.00%
	2)Variation between centrifuged sample PTFE 0.45 micron filters=0.00%
Intermediate precision	Individual impurity variation=0.03% Total impurity variation=0.08%

Table 10: Validation results of Bosentan pyrimidinone impurity in RS method

Parameter	**Results**
RRT	0.93 with respect to bosentan
Specificity	Blank interference, Placebo interference, Impurity interference was nil.
Linearity	Established from 1 ppm to 42 ppm (R^2 value=0.999)
LOD and LOQ	LOD=0.51 ppm and LOQ=1.54 ppm
Precision:	% RSD of impurity for six preparations= 1.44%
Accuracy	**%Level** **%Recovery**
	10% 100.8
	20% 98.9
	40% 105.1
	50% 104.5
	100% 100.0
	400% 98.5
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	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.00%
	2)Variation between centrifuged sample PTFE 0.45 micron filters=0.00%

Intermediate precision	Individual impurity variation=0.04% Total impurity variation=0.08%

Table 11: Validation results of Bosantan methoxy impurity in RS method

Parameter	Results
RRT	1.06 with respect to bosentan
Specificity	Blank interference, Placebo interference, Impurity interference was nil.
Linearity	Established from 1 ppm to 42 ppm (R^2 value=1)
LOD and LOQ	LOD=0.30 ppm and LOQ=0.91 ppm
Precision:	% RSD of impurity for six preparations= 0.79%
Accuracy	**%Level** **%Recovery** 10% 101.0 20% 96.9 40% 92.5 50% 95.5 100% 92.2 400% 92.9
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	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.01% 2)Variation between centrifuged sample PTFE 0.45 micron filters=0.01%
Intermediate precision	Individual impurity variation=0.03% Total impurity variation=0.08%

Table 12: Validation results of Bosantan BSN4FB impurity in RS method

Parameter	Results
RRT	1.07 with respect to bosentan
Specificity	Blank interference, Placebo interference, Impurity interference was nil.
Linearity	Established from 1 ppm to 42 ppm (R^2 value=0.999)
LOD and LOQ	LOD=1.80 ppm and LOQ=5.46 ppm
Precision:	% RSD of impurity for six preparations= 0.79%
Accuracy	**%Level** **%Recovery** 10% 104.4 20% 101.4 40% 100.7 50% 95.1 100% 95.8 400% 97.8
Solution stability	1)% Difference at 24 Hrs=0.04% 2)%Difference at 48 Hrs=0.04%
Robustness	Flow rate variation-System suitability passes Temperature variation system suitability passes
Ruggedness	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.02% 2)Variation between centrifuged sample PTFE 0.45 micron filters=0.02%
Intermediate precision	Individual impurity variation=0.02% Total impurity variation=0.08%

Table 13: Validation results of Bosantan Dimer impurity in RS method

Parameter	Results
RRT	1.11 with respect to bosentan
Specificity	Blank interference, Placebo interference, Impurity interference was nil.

Linearity	Established from 1 ppm to 42 ppm (R^2 value=1)
LOD and LOQ	LOD=0.59 ppm and LOQ=1.81 ppm
Precision:	% RSD of impurity for six preparations= 1.00%
Accuracy	**%Level** **%Recovery** 10% 96.3 20% 102.5 40% 96.8 50% 95.9 100% 92.5 400% 91.6
Solution stability	1)% Difference at 24 Hrs=0.01% 2)%Difference at 48 Hrs=0.03%
Robustness	Flow rate variation-System suitability passes Temperature variation system suitability passes
Ruggedness	1)Variation between centrifuged sample PVDF 0.45 micron filters=0.00% 2)Variation between centrifuged sample PTFE 0.45 micron filters=0.00%
Intermediate precision	Individual impurity variation=0.03% Total impurity variation=0.08%

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 1N Hydrochloric acid for 3 days and the degradation observed was 0.22% for sildenafil, and 0.71 for bosantan,In Impurities test the peak purity of Sildenafil,Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in acidic condition.

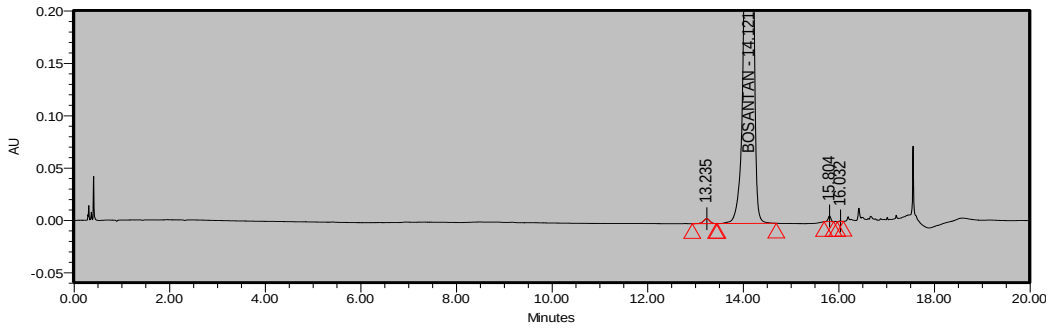

Figure 6: Acid degraded sample chromatogram-Bosentan

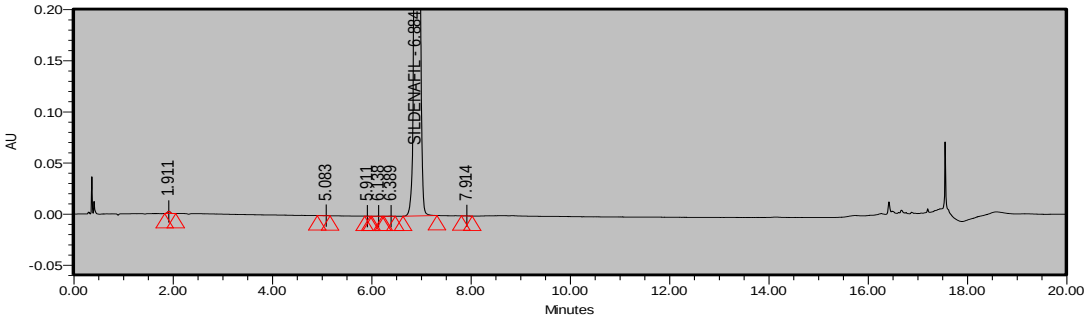

Figure 7: Acid degraded sample chromatogram-Sildenafil

Base degradation:

It was performed with 1N sodium hydroxide for 3 days and the degradation observed was 0.07% for sildenafil, and 0.69 for bosentan,In Impurities test the peak purity of sildenafil, bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in basic condition.

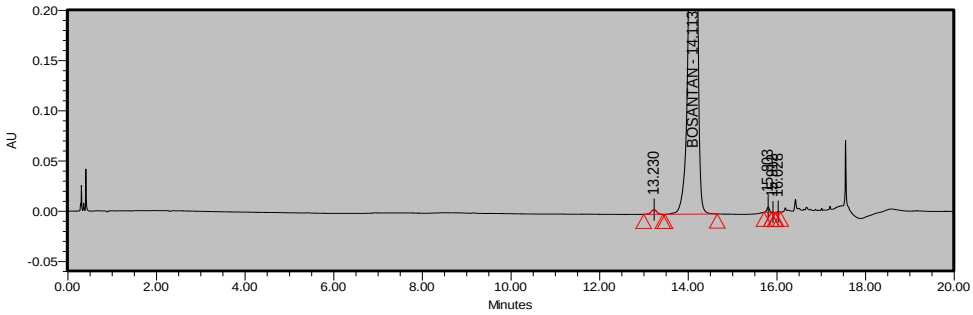

Figure 8: Base stressed sample chromatogram-Bosentan

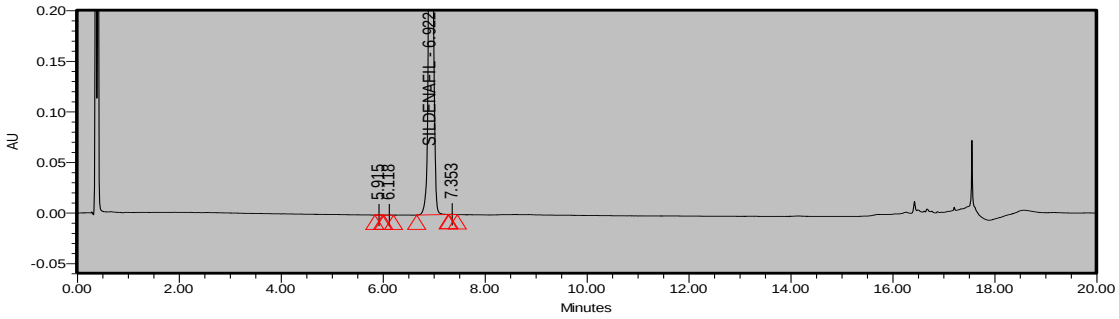

Figure 9: Base stressed sample chromatogram-Sildenafil

Peroxide degradation:

It was performed with 10% Hydrogen peroxide for 3 days and the degradation observed was 9.55% for sildenafil, and 0.65 for bosentan,In Impurities test the peak purity of Sildenafil, Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in peroxide condition.

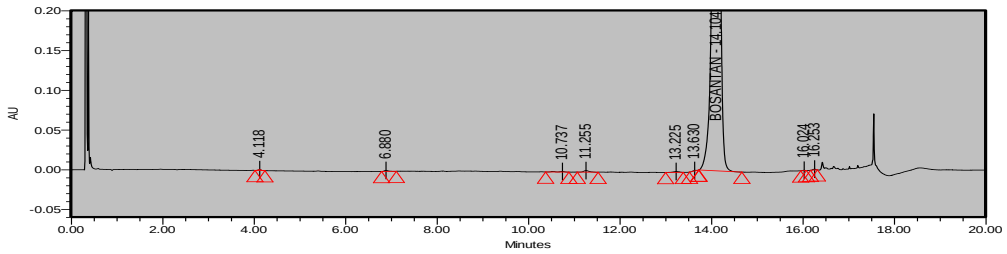

Figure 10: Peroxide stressed sample chromatogram-Bosentan

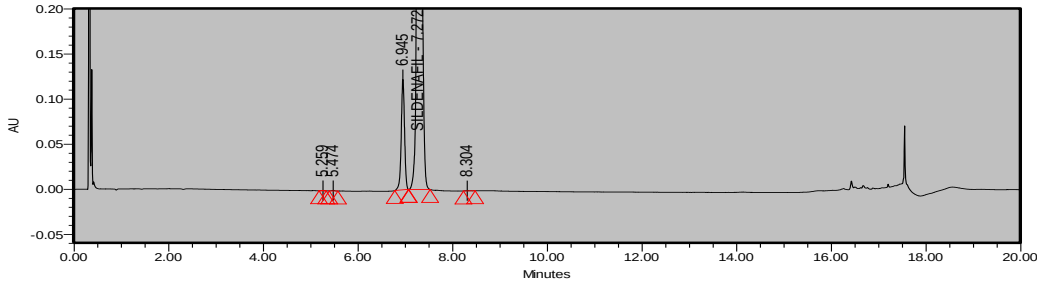

Figure 11: Peroxide stressed sample chromatogram-Sildenafil

Water degradation:

It was performed with milli Q Water for 3 days and the degradation observed was 0.34% for sildenafil, and 0.16% for bosentan,In Impurities test the peak purity of Sildenafil, Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in hydrolysis condition.

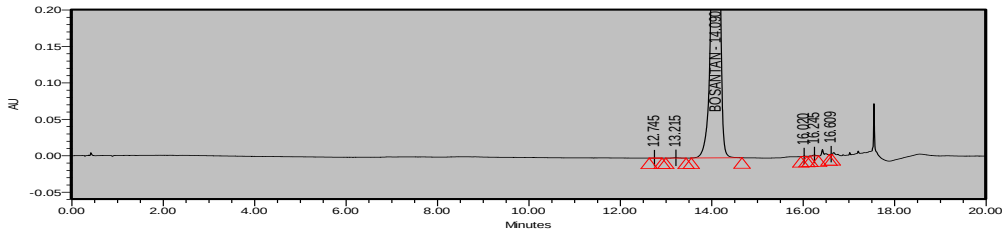

Figure 12: Water stressed sample chromatogram- bosentan

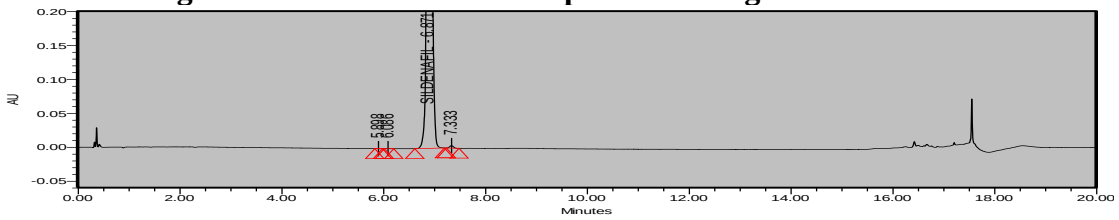

Figure 13: Water stressed sample chromatogram- Sildenafil

Thermal degradation:

It was performed at 50°C for 3 days and the degradation observed was 0.09% for sildenafil, and 0.12% for bosentan, In Impurities test the peak purity of Sildenafil, Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in thermal condition.

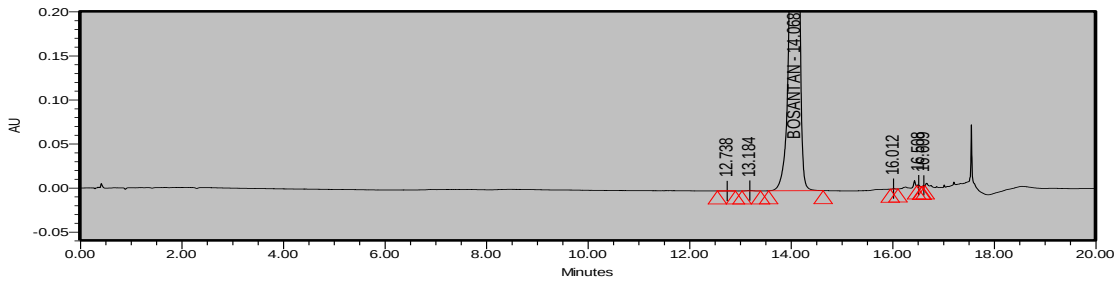

Figure 14: Thermal stressed sample chromatogram-Bosentan

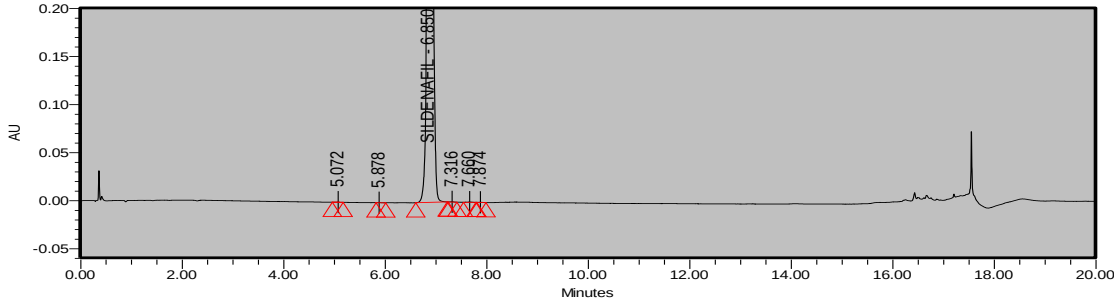

Figure 15: Thermal stressed sample chromatogram-Sildenafil.

Photo degradation:

It was performed by exposing the sample to1.2 million Lux hours and the degradation observed was 0.09% for sildenafil, and 0.17% for bosentan, In Impurities test the peak purity of Sildenafil, Bosentan and unknown and known impurities was passed, this proves that the method was stability indicating in thermal condition.

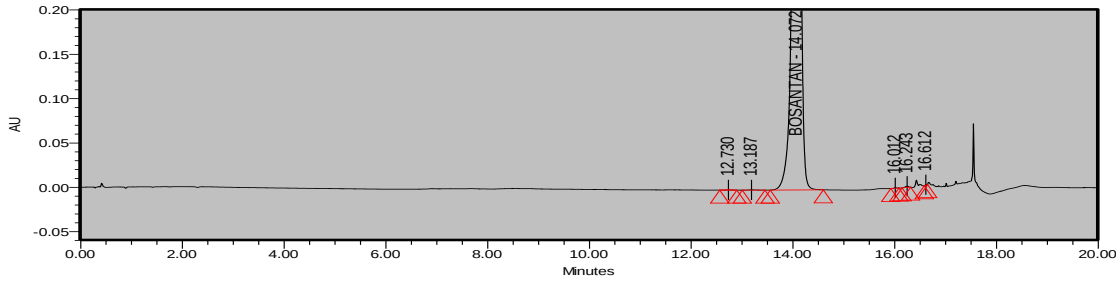

Figure 16: Light stressed sample chromatogram-Bosentan.

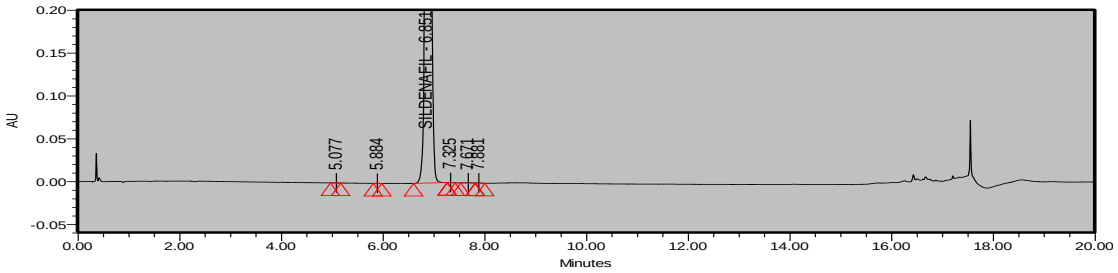

Figure 17: Light stressed sample chromatogram-Sildenafil

The overall summery of forced degradation results was as below.

Table 14: Forced degradation study results of sildenafil citrate

Type	Condition &Duration	% Degradation	%Assay	Peak purity
Acid	1N Hcl,3 days,50°C	0.22	99.74	Passes
Base	1N NaoH,3 days,50°C	0.07	99.92	Passes
Peroxide	10% H_2O_2,3 day,50°C	9.55	90.42	Passes
Water	Water,3 day,50°C	0.34	99.63	Passes
Thermal	3 days,50°C	0.09	99.9	Passes
Photo	1.2 million Lux hours	0.09	99.9	Passes

Table 15: Forced degradation study results of bosentan

Type	Condition &Duration	% Degradation	%Assay	Peak purity
Acid	1N Hcl,3 days,50°C	0.71	99.28	Passes
Base	1N NaoH,3 days,50°C	0.69	00.29	Passes
Peroxide	10% H_2O_2,3 day,50°C	0.65	99.31	Passes
Water	Water,3 day,50°C	0.16	99.84	Passes
Thermal	3 days,50°C	0.12	99.86	Passes
Photo	1.2 million Lux hours	0.17	99.83	Passes

System suitability equivalence:

The difference in the system suitability results between developed method and Pharmacopeia method was less The critical pair resolution was maintained above 2.0 in the developed method, it shows that the results are comparable to that of Pharmacopeia method results.

Table 16: System suitability equivalence table-Bosentan

Parameter	Pharmacopeia method	UPLC Method
Assay Standard %RSD	0.23	0.26
Critical pair resolution in impurities test	More than 1.5	More than 1.5

Table 17: System suitability equivalence table-Sildenafil

Parameter	Pharmacopeia method	UPLC Method
Assay Standard %RSD	0.35	0.32
Critical pair resolution in impurities test	More than 1.5	More than 1.5

API Batch analysis results equivalence:

The results obtained with the developed method was compared with the Pharmacopeia method results, the variation in assay and impurities results was below 0.1%, proves that the method was equivalent to the API Pharmacopeia method with 20 minutes runtime.

Table 18: Bosentan API Analysis results equivalence table

Details	Pharmacopeia 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	99.5%	4)99.6%

Table 19: Sildenafil citrate API Analysis results equivalence table

Details	Pharmacopeia method Results	UPLC Method results
B.No:SC0091011		
Known impurity	0.01%	0.01%
Any unknown individual impurity	0.02%	0.02%
Total impurity	0.08%	0.07%
Assay	100.0%	100.1%

Reference product analysis results equivalence:

The results obtained with the developed method shows the difference less than 0.05% with Pharmacopeia method, shows that the developed method is equivalent to that of Pharmacopeia method with 20 minutes Runtime.

Table 20: Reference product analysis (Bosantas-cipla) equivalence table

Details	API Vendor method Results	UPLC Method results
B.No:2345AF		
Known impurity	0.01%	0.01%
Any unknown individual impurity	0.03%	0.03%
Total impurity	0.21%	0.22%
Assay	100.1%	100.3%

Table 21: Reference product analysis (Viagra) equivalence table

Details	Pharmacopeia method Results	UPLC Method results
B.No:A236413		
Known impurity	0.01%	0.01%
Any unknown individual impurity	0.05%	0.05%
Total impurity	0.08%	0.09%
Assay	100.0	99.98

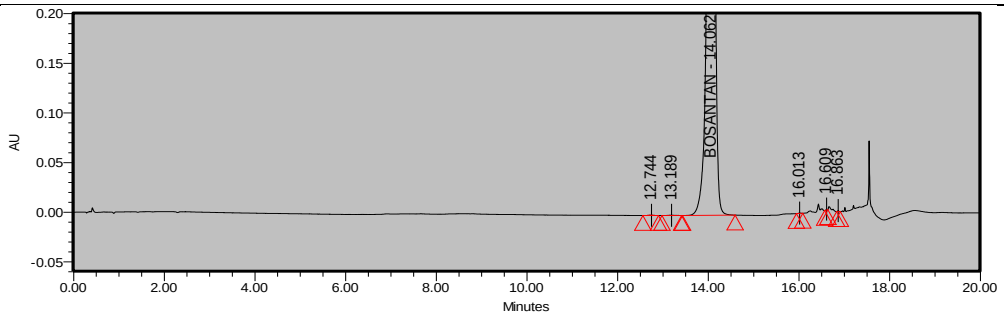

Figure 18: Bosantan sample chromatogram

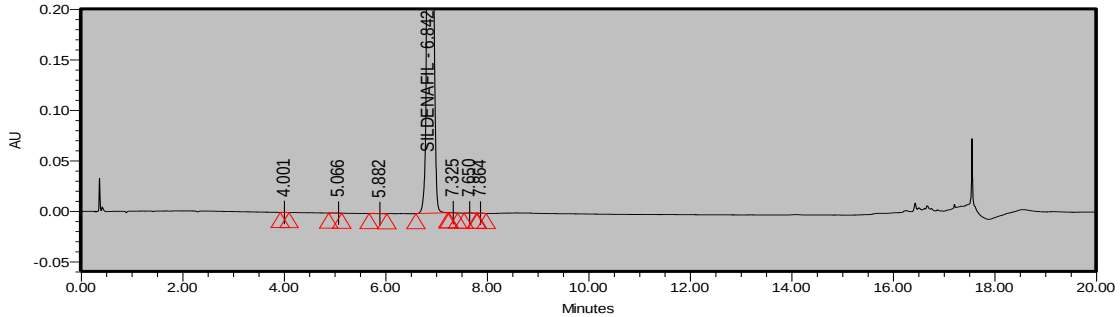

Figure 19: Viagra sample chromatogram

CONCLUSION:

An UPLC method for related compounds in the commercial drug products and in the tablet formulation was validated in this study. Sildenafil citrate, Bosentan, their degradents and impurities gave chromatograms of very well resolved peaks and mass balance above 99.5%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 Sildenafil citrate, Bosentan and its related impurities in bulk drugs and its tablet dosage forms for quality control purposes.

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