An Elsevier Indexed Journal ISSN-2230-7346

Journal of Global Trends in Pharmaceutical Sciences

DEVELOPMENT OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF BOSENTAN AND SILDENAFIL IN PURE AND PHARMACEUTICAL DOSAGE FORM

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ARTICLE INFO

Key Words
Bosentan, Sildenafil, RP-HPLC, Phosphate Buffer, Methanol, Validation.

Access this article online
Website: https://www.jgtps.com/

Quick Response Code:

ABSTRACT

A simple reverse phase HPLC method was developed for the simultaneous estimation of Bosentan and Sildenafil in bulk and tablet form. Chromatography was performed by isocratic reverse phase separation on a stainless steel column 4.6 x 150mm, symmetry column packed with octa decyl silane bonded to porous silica (C18) with particle size 5 micron with mobile phase containing phosphate Buffer of pH 3.0 and Methanol in proportion of 30:70 respectively. The flow rate was 1.0 ml/ min and effluent was monitored at 260 nm. The retention times were 2.66 min and 3.84 min Sildenafil and Bosentan respectively. The standard curve was linear over a working range of 50–250 µg/ml for Bosentan and Sildenafil and gave an average correlation coefficient of 0.999. The limit of quantitation (LOQ) of this method was 2µg/ml for Bosentan and Sildenafil. The absolute recovery was 99.75% for Bosentan and 99.12% for Sildenafil. Degradation products produced as a result of stress studies did not interfere with the detection of Bosentan and Sildenafil and the assay can thus be considered stability-indicating.

INTRODUCTION

Bosentan is a dual endothelin receptor antagonist important in the treatment of pulmonary artery hypertension (PAH). It is licensed in the United States, the European Union and other countries by Actelion Pharmaceuticals for the management of PAH under the trade name Tracleer. Bosentan is used to treat pulmonary hypertension by blocking the action of endothelin molecules that would otherwise promote narrowing of the blood vessels and lead to high blood pressure. Endothelin-1 (ET-1) is a neurohormone, the effects of which are mediated by binding to ET_A and ET_B receptors in the endothelium and vascular smooth muscle.[1]

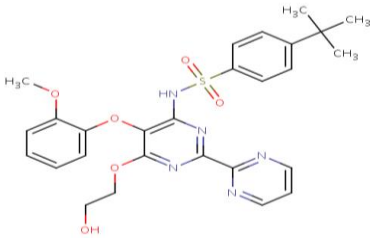

Fig no-1: Bosentan

ET-1 concentrations are elevated in plasma and lung tissue of patients with pulmonary arterial hypertension, suggesting a pathogenic role for ET-1 in this disease. Bosentan is a specific and competitive antagonist at endothelin receptor types ET_A and ET_B. Bosentan has a slightly higher affinity for ET_A receptors than for ET_B receptors.[1, 4]

Sildenfail is a vasoactive agent used to treat erectile dysfunction and reduce symptoms in patients with pulmonary arterial hypertension (PAH). Sildenafil elevates levels of the second messenger, cGMP, by inhibiting its breakdown via phosphodiesterase type 5 (PDE5). PDE5 is found in particularly high concentrations in the corpus cavernosum, erectile tissue of the penis. It is also found in the retina and vascular endothelium. Increased cGMP results in vasodilation which facilitates generation and maintenance of an erection. The vasodilatory effects of sildenafil also help reduce symptoms of PAH.

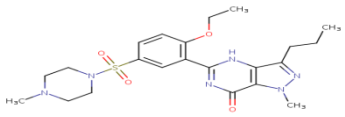

Fig No-2: Sildenafil

Sildenafil inhibits the cGMP-specific phosphodiesterase type 5 (PDE5) which is responsible for degradation of cGMP in the corpus cavernosum located around the penis. Penile erection during sexual stimulation is caused by increased penile blood flow resulting from the relaxation of penile arteries and corpus cavernosal smooth muscle. This response is mediated by the release of nitric oxide (NO) from nerve terminals and endothelial cells, which stimulates the synthesis of cGMP in smooth muscle cells. Cyclic GMP causes smooth muscle relaxation and increased blood flow into the corpus cavernosum. The inhibition of phosphodiesterase type 5 (PDE5) by sildenafil enhances erectile function by increasing the amount of cGMP.[2, 7, 8]

MATERIALS AND METHODS:

Drugs: Pure pharmaceutical sample of Bosentan(BSN) and Sildenafil (SFL) was obtained from Yucca Pharma. Commercial tablet of bosentan (62.5mg), Sildenafil (20mg) were procured from the local drug market.

Chemicals: Potassium dihydrogen phosphate (AR Grade), 85% Orthophosphoric acid (AR Grade), Methanol (HPLC Grade), Orthophosphoric acid (AR Grade), Triethyl-Amine (AR Grade), Sodium Hydroxide (AR Grade) were purchased from Sd fine-Chem limited. [3].

Instrument: Liquid chromatographic system from Waters alliance 2695 with Waters UV detector equipped with Empower software was used.

Preparation of mobile phase: Accurately measured 300 ml (30%) of above buffer and 700 ml of Methanol HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.. [5]

Preparation of Phosphate buffer: Accurately weighed 6.8 grams of KH_2PO_4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.0 with Orthophosphoric acid.

Diluent Preparation: The Mobile phase was used as the diluents**.**

Stock solutions and standards: Accurately weigh and transfer 100 mg of Bosentan and Sildenafil 100 mg of working standard in to a 100ml clean dry volumetric flask add about 70mL of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Preparation of Sub Stock Solution: Further pipette 1 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent..

Preparation of sample solution: Accurately weigh 10 tablets crush in mortar and pestle and transfer equivalent to

100 mg of Bosentan and 100 mg of Sildenafil (marketed formulation) sample into a 100mL clean dry volumetric flask add about 70 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 1.5 ml of Sildenafil and 1.5 ml of Bosentan from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Stability Study: Tablet powder equivalent to the weight one tablet was transferred to 250 ml round bottomed flask and treated under acidic, alkaline, oxidizing, thermal and photolytic stress conditions. When degradation was complete, the solution were left to equilibrate to room temperature and diluted with diluents to furnish solutions of concentration equivalent to 150 µg/ml Bosentan and 150µg/ml Sildenafil. The specific conditions are

described below. In acidic degradation drug was heated under reflux with 1M hydrochloric acid for 60 min at 60° C. For alakaline degradation drug was treated with 1N NaOH at 60° C for 1 h. The drug was treated with 5% (v/v) H_2O_2 at room temperature for 2 hour in oxidative degradation. Thermal degradation was performed by exposing the solid drug to dry heat in a convection oven at 70°C for 24 h and photolytic degradation was performed by exposing the drug to sunlight for 24 hour.[4]

Apparatus and Chromatographic conditions: Quantitative HPLC was performed on Waters HPLC system with UV detector. empower software is used along with a stainless steel column 4.6 x 150mm, packed with Octa decyl silane bonded to porous silica (C18) with particle size 5 micron. To develop a suitable and robust HPLC method for the determination of BSN and SFL, different mobile phases containing buffer and Methanol were used in different compositions like (30:70, 40:60, 50:50, 70:30, 80:20) at different flow rates (0.5,0.75,1.0, 1.2, 1.5, ml/min). The mobile phase containing buffer and methanol with a flow rate of 1.0 ml/ min gave peaks of good resolution and were eluted at retention times around 2.66 min, 3.84 min with symmetric peak shape. The detection is performed at the wavelength 260 nm [3, 6, 7, 13, 14]

Running the standard solution of Bosentan and Sildenafil: 1.5 ml of BSN stock solution and 1.5 ml SFL stock solution was pipetted into a 100 ml volumetric flask. The volume was made up to the mark with mobile phase. The solution was filtered through the 0.45 µm membrane filter and degassed under ultrasonic bath prior to use. The solution was injected into the HPLC system. The chromatogram obtained is shown in figure 5.

RESULTS AND DISCUSSION:

Method development and optimization: The main target of the chromatographic method is to get the separation of closely eluting drugs Bosentan and Sildenafil, The drugs were co-eluted by using different stationary phases like C18, C8 with varying lengths and different mobile phases containing buffers like phosphate, sulphate and acetate with different pH (2-7) and using organic modifiers like acetonitrile, methanol and ethanol in the mobile phase. pH of the buffer has played a significant role in achieving the separation between drugsl. The chromatographic separation was achieved on a stainless steel column (4.6 x 250mm) column packed with Octa decyl silane bonded to porous silica (C18) with particle size 5 micron, by using solutions phosphate buffer and methanol in the ratio of (30:70), pH adjusted to 3 using ortho phosphoric acid. The flow rate of the mobile phase was maintained at 1.0 ml/min. At 25^0 C of column temperature, the peak shape of BSN and SFL was found symmetrical with mobile phase 30:70 ratio. In the optimized conditions BSN and SFL were well separated with a good resolution and the typical retention times of SFL and BSN were about 2.6 min and 3.8 min, respectively. The system suitability results are given in table no.1 and the developed LC method was validated.[3,8, 9, 10, 11, 12,]

Results of method validation

Linearity: Linear calibration plot for assay method was obtained over the calibration ranges tested, i.e. 50 to 250 µg/ml for Bosentan and Sildenafil , the correlation coefficient obtained was greater than 0.999. The results show that an excellent correlation existed between the peak area and concentration of the analyte which is given in table 3 and 4.

Intermediate precision/ruggedness: To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.

Recovery and accuracy: The percentage recovery of Bosrntan and Sildenafil in bulk drugs samples was ranged from 99.84 – 100.51% which indicates that the method was accurate which is given in table no.7.

Accuracy results: The accuracy of the method was determined by preparing solutions of different concentrations of BSN and SFL that is 80%, 100% and 120% in which the amount of marketed formulation (BSN and SFL 150 mg was kept constant and the amount of pure drug was varied that is 120 mg, 150 mg and 180 mg for SFL and BSN. The Solution was diluted to get concentration in linearity range of 120 µg, 150 µg, 180 µg respectively for 80%, 100%

and 120% respectively. The solutions were prepared in triplicates and the accuracy. Similarly was indicated by % recovery in table 7 and 8.

Specificity: 10 mg/ml of BSN was spiked with 50% (5 mg), 100% (10 mg),and 150% (15 mg) of excipient mix (Magnesium Stearate), Further 1.5 ml is pippeted out from the all three samples and diluted to 100 ml in three separate volumetric flask, and analysed for % recovery of BSN.Similarly 10 mg/ml SFL sample were prepared and analysed.

ROBUSTNESS: Robustness is a measure of capacity of a method to remain unaffected by small, but deliberate variations in the method conditions, and is indications of the reliability of the method. A method is robust, if it is unaffected by small changes in operating conditions. To determine the robustness of this method, the experimental conditions were deliberately altered at three different levels and retention time and chromatographic response were evaluated. One factor at a time is mobile phase flow rate by 0.1 ml/min (0.9 and 1.1ml/min) had no significant effect on the retention time and chromatographic response of the 150 µg/ml solution, indicating that the method was robust. Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Change in content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-19, % RSD < 2%) the developed RP-HPLC method for the analysis of Sildenafil and Bosentan.

Acceptance criteria: Percentage RSD should be below 2.The %RSD obtained for change of flow rate, variation in mobile phase was found to be below 1, which is within the acceptance criteria. Hence the method is robust

Stability Studies

Forced degradation studies: Forced degradation of Test sample was performed under acidic, alkaline, heat, photolytic and oxidative stress conditions.

***Stock solution preparation*:** Twenty Tablets were weighed and powdered. Tablet powder having weight equivalent to 100 mg of was weighed accurately and taken in a 100 ml volumetric flask. To it 50 ml of the mobile phase was added and sonicated for 15 minutes to dissolve the drugs. The volume was made up to 100 ml with mobile phase. The resulting solution was then filtered through a 0.45 µm membrane filte0r to prepare a stock solution of the tablet sample. Further dilution was done by diluting 1.5 ml of stock solution to 100 ml mobile phase. The concentration of Sildenafil and Bosentan in the solution was 150 µg/ml.

I. Acid Hydrolysis: Forced degradation in acidic media was performed by adding 2 ml 1 M HCl to 10 ml of stock solution and the mixture is heated at 60°C for approximately 1 hour. After neutralization the prepared solution is injected and chromatograms were recorded.

Observation: The study indicates that the drugs under study were degraded and assay results shows that mostly Sildenafil (3.7%) was degraded following by Bosentan (3.4%) and no significant peak was observed for degradants.

II. Basic Hydrolysis: Forced degradation in basic media was performed by adding 2 ml 1 M NaOH to 10 ml of stock solution and the mixture is heated at 60°C for approximately 1 hour and the solution is neutralized by addition of 1 M HCl. The prepared solution is injected and chromatograms were recorded.

Observation: The results of alkaline hydrolysis indicates that some degradation occurred and assay results shows that Sildenafil was degraded by 1.3% and Bosentan was degraded by 3.8% and no significant peak was observed for degradants.

III. Dry Heat Degradation: To study the effect of temperature an aliquot of stock solution was kept at 70^0C for 24 hrs. 20 µl of resulting solution was injected into HPLC and chromatograms were recorded

Observation: From the observation it was found that both Sildenafil and Bosentan were found stable i.e, no significant peaks were found. So it is stable in the above condition.

IV. Photolytic Degradation: To study the effect of photolysis, an aliquot of stock solution was exposed to UV light for 24hrs. 20µl of resulting solution was injected into HPLC and chromatograms were recorded.

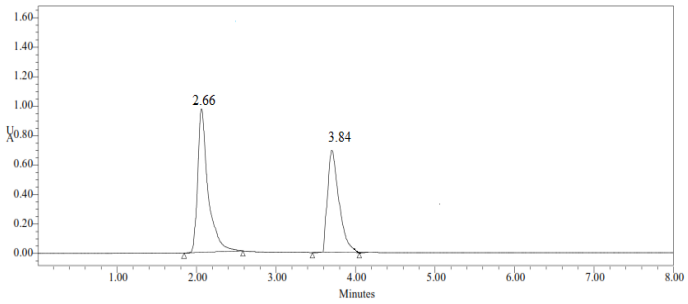

Fig No -3 chromatogram of Sildenafil (Rt-2.66 min) and Bosenta (Rt-3.84 min)

S.No	Name	Retention time(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Sildenafil	2.66	918096	201430	6 0	1.2	4673.4
2	Bosentan	3.84	1208022	140319	6 0	1.3	6090.3

Table No-1: System suitability parameters

Instrument used	: Waters HPLC with auto sampler and UV detector
Temperature	Ambient
Column	Inertsil C18 (4.6 x 250mm, 5µm)
Buffer :	Accurately weighed 6.8 grams of KH2PO4 dissolved 1000ml. volume was adjusted to pH 3.0 with Orthophosphoric acid.
pH	3
Mobile phase	30% buffer 70% Methanol
Flow rate	1 ml per min
Wavelength	260 nm
Injection volume	20 µl
Run time	10 min

Table-2 Analytical performance parameters of Sildenafil and Bosentan

Parameters	Bosentan	Sildenafil
Slope (m)	402892	306198
Correlation coefficient (R2)	0.999	0.999

Table no -3: Results of method precession for Sildenafil

S. No.	Peak name	Rt	Area (µV*sec)	USP Plate Count	USP Tailing
1	Sildenafil	2.65	918096	1.0	3802
2	Sildenafil	2.66	918090	1.1	3546
3	Sildenafil	2.67	916996	1.4	4633
4	Sildenafil	2.66	915986	1.1	4812
5	Sildenafil	2.65	916076	1.0	3802
	Mean		917149.67		
	Std. Dev		955.47		
	% RSD		0.10		

Table no - 4: Results of method precession for Bosentan

S.No.	Peak Name	Rt	Area (µV)	USP Tailing	USP Plate Count
1	Bosentan	3.81	1208918	1.2	4759
2	Bosentan	3.82	1208414	1.1	3695
3	Bosentan	3.83	1208518	1.1	4741
4	Bosentan	3.84	1208309	1.2	3793
5	Bosentan	3.84	1208105	1.1	4741
	Mean		1208424		
	Std. Dev.		278.81		
	% RSD		0.02		

Table No. 5 Accuracy studies for Sildenafil (150 mcg)

% Concentration (at specification Level)	Area	Amount Added (µg)	Amount Found (µg)	% recovery	Mean Recovery
80%	478001	120	118.8	99.0%	99.12%
100%	918096	150	148.6	99.06%	
120%	1106240	180	178.7	99.3%	

Table-6: Accuracy results for Bosentan(150 mcg)

% Concentration (at specification Level)	Area	Amount Added (µg)	Amount Found (µg)	% recovery	Mean Recovery
80%	628055	120	119.24	99.37%	99.75%
100%	1208022	150	149.25	99.5%	
120%	1455579	180	180.07	100.4%	

Table No. 7: Results of specificity studies for Bosentan and Sildenafil

LOD and LOQ: The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio.

Drug name	**Baseline noise(µV)**	**Signal obtained (µV)**	**S/N ratio**
Sildenafil	52	522	10.03
Bosentan	52	524	10.1

- Signal to noise ratio shall be 10 for LOQ solution, The result obtained is within the limit.

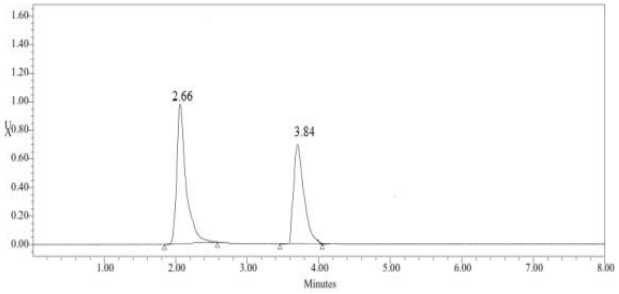

Fig - 4: Chromatogram showing degradation for Bosentan and Sildenafil in 0.1 N HCl

Table No.8: Results of robustness for Sildenafil

%Concentration (at specification Level)	Area	Drug Added (µg)	Excipient Added (µg))	Amount Found (µg)	% Recovery	Mean Recovery
50%	918086	150 mg	75 mg	149.9 mg	99.6%	99.6%
100%	918096	150 mg	150 mg	149.9mg	99.7%	
150%	918076	150 mg	225 mg	149.9 mg	99.9%	
Specificity data for Sildenafil						
%Concentration (at specification Level)	Area	Drug Added (µg)	Excipient Added (µg))	Amount Found (µg))	% Recovery	Mean Recovery
50%	1207999	150	75	149.8 mg	99.4%	99.5%
100%	1208022	150	150	149.9mg	99.6%	
150%	1208003	150	225	149.9 mg	99.6%	

Flow Rate (ml/min) data for Sildenafil

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	5339.9	1.4
2	1.0	4673.4	1.3
3	1.1	5216.0	1.4

Change in Organic Composition in the Mobile Phase for Sildenafil

S.No	Change in Organic Composition in the	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	4508.4	1.3
2	*Actual	4673.4	1.4
3	10% more	4318.1	1.3

Table No.9: Results of robustness for Bosentan

Flow rate (ml/min) data for Bosentan

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	7063.3	1.3
2	1.0	6090.3	1.2
3	1.1	6998.0	1.3

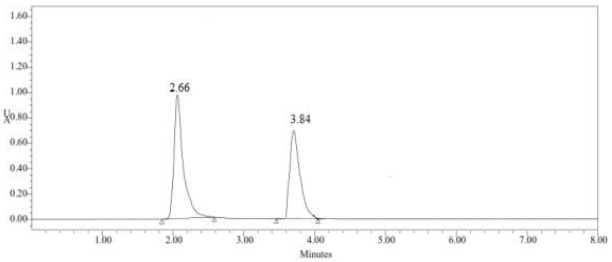

Fig No.-5: Chromatogram showing degradation related impurity in 0.1 N NaOH

Change in Organic Composition in the Mobile Phase for Bosentan

S.No		Change in Organic Composition in the		System Suitability Results		
				USP Plate Count		USP Tailing
1		10% less		6387.7		1.2
2		*Actual		6090.3		1.2
3		10% more		6232.5		1.2
S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Sildenafil	2.66	884126	197831	1.2	4854
2	Bosentan	3.84	1154879	145461	1.3	3872

S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Sildenafil	2.66	906161	196833	1.0	4658
2	Bosentan	3.84	1162137	146372	1.1	3694

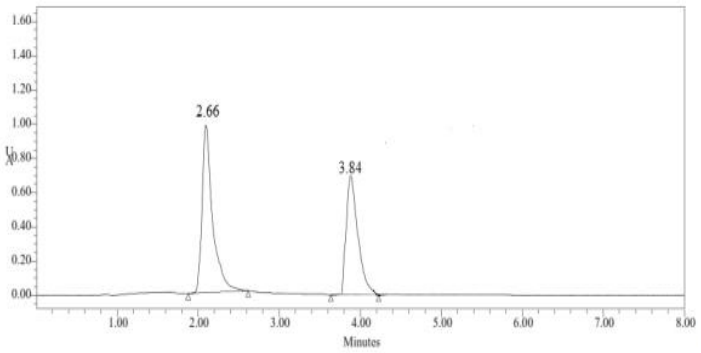

Fig 6: Chromatogram showing thermal degradation studies

S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Sildenafil	2.66	902488	196600	1.2	4821
2	Bosentan	3.84	1151254	148991	1.4	3365

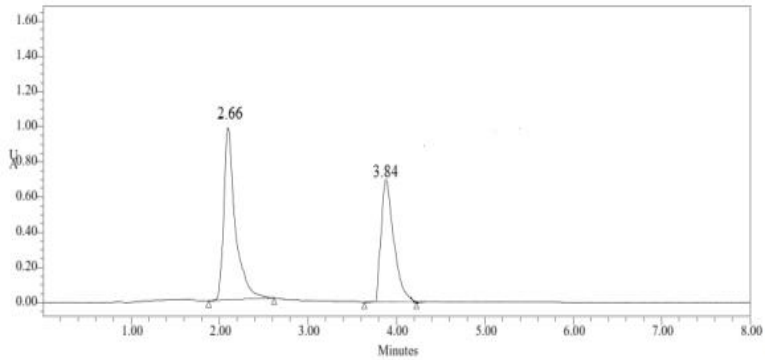

Fig No. -7: Chromatogram of Photolytic degradation of sample

S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Sildenafil	2.66	901570	197831	1.0	4857
2	Bosentan	3.84	1186267	145461	1.2	3635

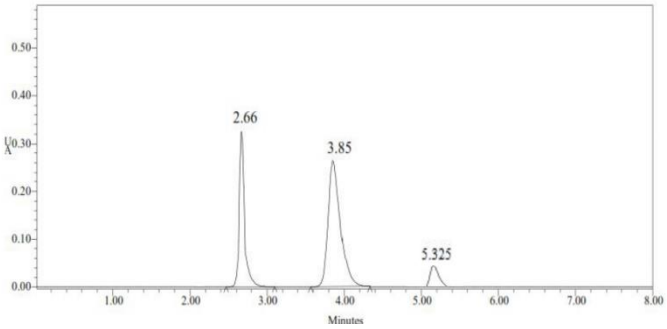

Fig-8: Chromatogram shows oxidative degradation.

S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Sildenafil	2.66	1120882	169910	1.2	4635
2	Bosentan	3.85	1061831	139461	1.5	3458

Table -10: Results of forced degradation studies of Sildenafil and Bosentan API

Stress Condition	Sample-1 (Bosentan)			Sample-2 (Sildenafil)		
	Area	%Assay	%Degradation	Area	%Assay	%Degradation
Acidic	1154879	95.6	3.4	884126	96.3	3.7
Alkaline	1162137	96.2	3.8	906161	98.7	1.3
Photolytic	1186267	98.2	1.8	901570	98.2	1.8
Thermal	1151254	99.0	1.0	902488	98.5	1.5
Oxidative	1061831	87.9	12.1	818024	89.1	10.9

Observation: From the observation it was found that both Sildenafil and Bosentan were found stable i.e, no significant peaks were found. So it is stable in the above condition.

V. *Oxidative Degradation:* To study the effect of oxidizing conditions, an aliquot of stock solution was added to 10 ml 5 % H_2O_2 solution. The solution was shaken for one hour at 60^0C. The prepared solution 20µl was injected and chromatograms were recorded.

Observation: From the observation it was found that both Sildenafil and Bosentan were found degraded by 10.9% and 12.1% respectively

Results of forced degradation studies: The results of the stress studies indicated the **specificity** of the method that has been developed. Sildenafil and Bosentan were stable in photolytic, thermal and basic stress conditions. The result of forced degradation studies are given in the following table 2.

CONCLUSION:

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Sildenafil and Bosentan was done by RP-HPLC. The proposed method was found to be simple, precise, accurate and rapid for determination of SFL and BSN in pure and dosage form. The mobile phase is simple to prepare and economical. The sample recoveries in all formulations were in good agreement within the limit. Hence, this method can be easily and conveniently adopted for routine analysis of SFL and BSN in pure form and dosage form.

Acknowledgements: I, Dhiraj Kumar, thankful to Dr. T. Rama Rao, Associate Director, School of Pharmacy, GNITC Campus, Ibrahimpattnam, Hyderabad, for providing necessary facilities to carry out the research work.

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