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DEVELOPMENT OF VALIDATED RP- HPLC METHOD FOR BOSENTAN IN FORMULATION AND ITS APPLICATION TO *IN-VITRO* INTERACTION STUDY WITH ACECLOFENAC

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ABSTRACT

A simple, specific and selective internal standard method using RP-HPLC – PDA was developed for the determination of bosentan in its tablet dosage form. Chromatographic method was achieved on C18 column using a mobile phase system consisting of methanol: 0.1% formic acid pH 6.4 (70:30, v/v). Linearity was found in the concentration range of 1-6µg/mL. Low relative standard deviation and good % recovery values of the method showed that the developed method was highly precise, accurate and free from interference present in formulation. The method was successfully applied to the *in-vitro* interaction study because protein binding is one of the important pharmacokinetic parameters of a drug. Simultaneous administration of two or more drugs can modify the interaction of the drug to plasma protein and in turn percentage of protein binding. Drug-drug interaction alters the known therapeutic response of the drug and there may be enhanced or diminished effects of one or both drugs. The binding interaction of bosentan with aceclofenac was studied under simulated physiological condition using RP-HPLC. The study involved development and validation RP-HPLC for the determination of bosentan in presence of aceclofenac, finding the saturation concentration of bosentan determination of equilibration period and *in-vitro* interaction of bosentan with aceclofenac. Highest percentage protein binding of bosentan at saturation level was found to be 89.45%. From the interaction study it was observed that there is significant decrease in bosentan may lead to increase in their plasma concentration. Therefore, it may be concluded that bosentan should not be co-administered with aceclofenac.

Key words: Bosentan, RP- HPLC, Analysis of formulation, Interaction, Aceclofenac, ICH guidelines.

INTRODUCTION

Bosentan is a pulmonary arterial hypertensive drug.[1] Chemically it is 4-tert-butyl-N-[6-(2-hydroxy ethoxy)-5-(2-methoxy phenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl] benzene-1-sulfonamide with empirical formula $C_{27}H_{29}N_5O_6S$ (Fig.1). Whereas aceclofenac is used as non steroidal anti inflammatory drug, chemically it is 2-[2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl]oxyacetic acid.[2] Literature survey revealed that bosentan was estimated by external standard method, in presence of its decomposition products and metabolites using RP-HPLC[3-6] and LC-MS.[7] Using internal standard method, estimation of aceclofenac and the simultaneous determination was done by RP-HPLC.[8,9] Present study of estimation of bosentan was carried out using aceclofenac as internal standard which is comparatively more specific and accurate. The method has been optimized and validated as per the ICH guidelines. [10]Apart from this the method was applied to *in vitro* drug- drug interaction. Plasma protein binding properties are considered as the primary determinants of the pharmacokinetic properties of drugs. [11,12] Any physiological condition that causes alteration in the albumin binding of drugs might lead to change in the pharmacokinetic and pharmacological properties of the drugs. Drug- drug interactions thus play a vital role in the extent of plasma protein binding and consequently the therapeutic effect of drugs. Literature survey reveals that bosentan and aceclofenac are highly protein bound (more than 98% preferably to albumin fraction). So when they are co- administered there may be competitive binding and hence interaction may take place. Here the *in vitro* protein binding of bosentan has been conducted by equilibrium dialysis method using RP-HPLC method. In this study, the free fraction of drugs and the % of protein binding of bosentan to BSA in the presence and absence of aceclofenac were calculated. This study was done to evaluate the interaction of aceclofenac with bosentan at physiological pH and temperature (37±0.5˚C).

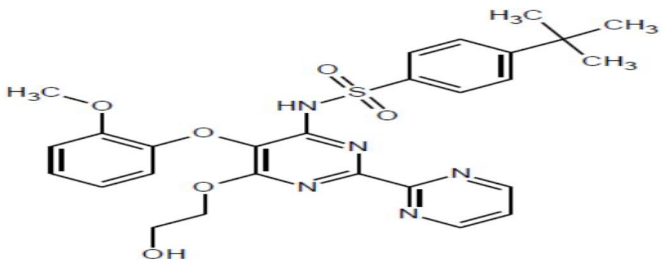

Fig. 1: Chemical structure of bosentan

MATERIALS AND METHODS

Materials

Bosentan was procured from MSN Laboratories Pvt. Ltd., Hyderabad, India, was used without further purification. Methanol, Formic acid and water used were of HPLC grade and were purchased from Merck India. The liquid chromatograph mass spectrometer Shimadzu HPLC -2010 EV System, which consisted of following components: a binary gradient pump variable wavelength programmable PDA detector with an auto sampler system. The chromatographic analysis was performed using Compaq Intel Core-2 DUO HP W/907 software on a pre-packed RP-18 column (250×4.6 mm, 5 µm particle size). In addition, an electronic balance (Shimadzu. Elec.balance BL-220H), a pH meter (Elico L127), a sonicator (Leclasonic ultrasonic cleaner), a hot air oven (Inlab equipments Ltd) were used in the study

HPLC Method [13, 14]

Selection of chromatographic mode of separation

Proper selection of method depends upon the nature of sample, its molecular weight and solubility. Since bosentan is polar in nature, RP-HPLC method with C18 column was used for the development of the method.

Preparation of stock and standard solution

Standard stock solution of bosentan and aceclofenac (100µg/mL) were prepared in methanol. Stock solution was diluted with mobile phase to get a series of concentrations containing 1-6 µg/ml of bosentan and 2µg/ml of aceclofenac.

Preparation of sample solution

Ten tablets, each containing 62.5 mg of bosentan were weighed and average weight was calculated. Weight equivalent to 10 mg of bosentan was weighed, transferred to 100 mL standard flask, extracted with methanol and made up with the same solvent, this solution was filtered through Whatmann filter paper and suitable aliquots of formulation solutions were prepared.

Fixed chromatographic condition

By considering few parameters like polarity, solubility and absorption maximum the following chromagraphic conditions were selected as

Stationary phase : Merk- LichroCART, C18 column (250 mm×4mm, 5µm)

Mobile phase : Methanol: 0.1% formic acid (pH-6.4)
Solvent ratio : 70: 30, v/v
Detection wavelength : 275 nm
Flow rate : 1 mL/min
Temperature : Room temperature

RESULTS AND DISCUSSION

Selection of wavelength

Selectivity of HPLC method that uses UV detector depends on proper selection of wavelength. A wavelength which gives good response for the drug to be detected is to be selected. From the UV spectra 275 nm was selected as detection wavelength, fig.2.

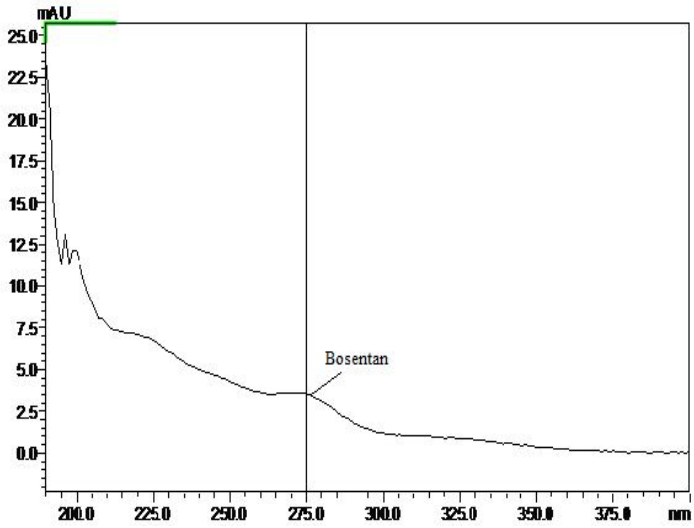

Fig. 2: Spectrum of standard bosentan

Selection of mobile phase

Solvent selectivity (solvent type), solvent strength (percentage of organic solvent in the mobile phase), strength and pH of buffer, flow rate etc. were optimized to determine the chromatographic conditions, that gave the best separation (Table 1).

Table 1: Selection of mobile phase

Mobile phase	Observation
Methanol: Water	Broad peak
Acetonitrile: Water	Broad peak
Methanol: Water(pH-6.2)	Tailing
Methanol: 10mM ammonium formate	Peak shape is not good
0.1% formic acid: Methanol(pH- 4.4)	Peak shape is not good
0.1% formic acid: Methanol	**Acceptable peak**

Selection of ratio of mobile phase

In a mobile phase system consisting 0.1% formic acid and methanol (pH adjusted to 6.4) different ratios like 20:80, 30:70 %v/v a mixture of bosentan and aceclofenac (internal standard) were injected (Table 2). Symmetrical peaks with good resolution was obtained with a ratio of 30:70%v/v and hence selected for further studies.

Table 2: Selection of ratio of mobile phase

0.1% Formic acid: Methanol (pH- 6.4)	Retention time (min)	
	Bosentan	Aceclofenac
30:70	**6.2**	**8.5**
20:80	3.9	4.5

Effect of pH

Keeping the ratio of mobile phase constant (70:30 % v/v), the chromatograms were recorded with different pH like 3.5, 4.4 and 6.4 were tried. For pH of 6.4, good resolution and symmetrical peak was obtained and hence selected for further studies (Table 3).

Table 3: Selection of pH

pH	Tailing factor	Observation
3.5	2.0	Broad peak, Tailing
4.5	1.6	Broad peak
6.4	**1.2**	**Good peak shape**

Selection of flow rate

Keeping all the parameters of mobile phase system constant, the chromatograms were recorded with different flow rates like 0.8, 1and 1.2ml/min, with flow rate 0.8 and 1.2, peaks were not symmetrical. But a flow rate of 1mL/min gave good symmetrical peaks and hence selected for further studies (Table 4).

Table 4: Selection of flow rate

Flow rate (ml/min)	Retention time (min)		Observation
	Bosentan	Aceclofenac	
0.8	6.5	8.4	Slight fronting
1	**6.2**	**8.2**	**Good peak shape**
1.2	5.9	7.0	Tailing

Selection of strength of formic acid

Different ionic strengths of formic acid such as 0.05%, 0.1%, 0.5% etc. in the ratio 30:70, v/v with methanol were tried. Good peak characteristics was observed for strength of 0.1% and hence selected for further study (Table 5).

Table 5: Selection of strength of formic acid

Strength (%)	Observation
0.05	Fronting
0.1	**Good**
0.5	Tailing

Validation

Specificity

Conditions of HPLC method like percentage of organic solvent in mobile phase, ionic strength, pH of buffer, flow rate etc., were changed. Although these changes were made, no additional peaks were found but there were some slight changes in retention time and peak shapes. Peak purity tests were done. The peak purity index of bosentan was found to be 0.9999. Peak purity index values close to one proves peak purity of the drug.

Linearity and range

Calibration graph was plotted using peak area ratios of bosentan and aceclofenac Vs concentration of standard solution. Linear regression data revealed an excellent linear relationship in the concentration range of 1-6 µg/mL (Fig.3). The slope intercept and correlation co-efficient values were found to 0.4273, -0.00414, 0.9996 respectively (Fig.4).

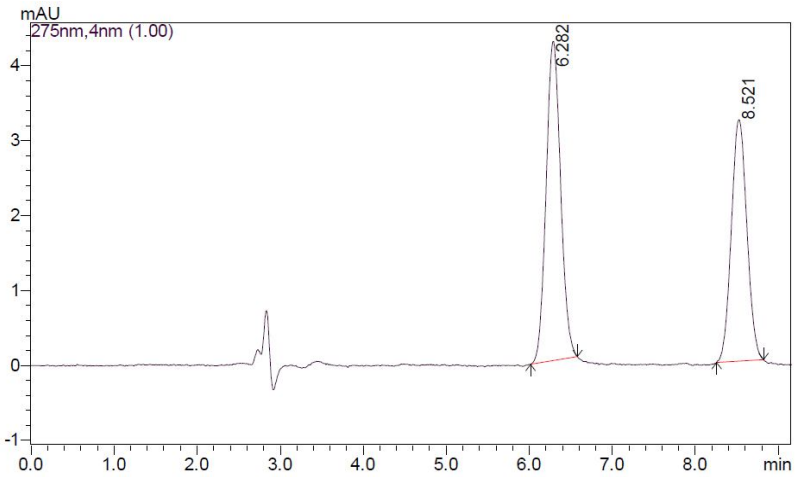

Fig. 3: Standard chromatogram of Bosentan (3 µg/mL) and Aceclofenac (2 µg/mL)

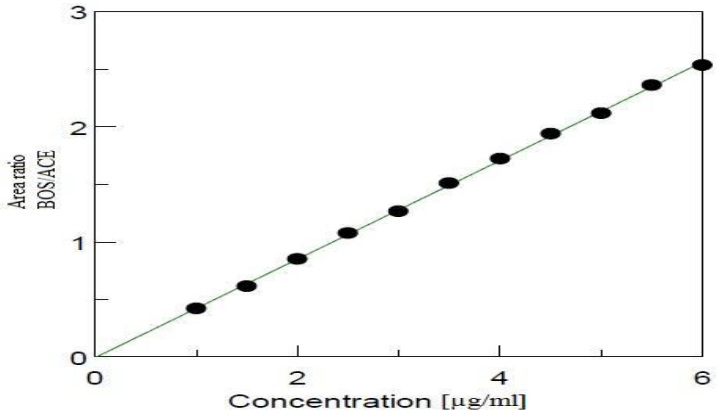

Fig. 4: Calibration graph of bosentan (1-6 µg/mL)

Accuracy

Recovery studies were done for determining accuracy parameter. It was done by mixing known quantity of standard drug with the analysed sample formulation and the contents were reanalyzed by the proposed method. Recovery studies carried out at 50 and 100% levels. The percentage recovery and its %RSD were calculated (Table 6).

Table 6: Recovery studies

Level	% Recovery	% RSD*
50%	100.11	0.518
100%	99.53	1.137

**Average of six determinations*

Precision

Intraday and interday precision were done by carrying out analysis of standard drug solutions at two different concentrations in the linearity range (2.5, 3µg/mL) were as for the repeatability study 3µg/mL was used and were analysed for six times on the same day for intraday and repeatability study. For interday the study was conducted six days over a period of one week. %RSD was calculated (Table7).

Table 7: Precision

Concentration (µg/mL)	Average peak area ratio Bosentan/Aceclofenac	%RSD*
Intraday		
2.5	1.0307	0.7478
3	1.0890	0.7294
Interday		
2.5	1.0280	0.3077
3	1.0912	0.4572
Repeatability of injection		
3	1.0908	0.1522

**Average of six determinations*

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ were determined by injecting progressively lower concentrations of the drug. LOD and LOQ of bosentan were found to be 0.1 µg/mL and 0.4 µg/mL respectively.

Robustness

In order to demonstrate the robustness of the method, the ratio of methanol in mobile phase, pH of buffer and flow rate were slightly varied from the optimised condition (Table 8).

Table 8: Robustness

Chromatographic condition		Peak area ratio Bosentan/Aceclofenac	Resolution
Mobile phase ratio (Methanol:0.1%Formicacid) pH 6.4	69:31	1.0422	5.92
	71:29	1.0459	5.96
pH of mobile phase	6.3	1.0375	5.74
	6.5	1.0213	5.87
Strength of formic acid	0.9%	1.0255	5.90
	1.1%	1.0281	5.84
Flow rate(ml/min)	0.9	1.0482	5.76
	1.1	1.0654	5.89

System suitability studies

System suitability parameters like number of theoretical plates, peak asymmetry factor, resolution and tailing factor were studied from the chromatographic peak, the results are given in table 9.

Table 9: System suitability studies

Number of theoretical plates	Asymmetric factor	Resolution	Tailing factor
52515	1.02	5.901	1.02

Stability

Sample solution of bosentan was subjected to stability studies under refrigeration and room conditions. Stabilities were studied by looking for any change in retention time, resolution, peak shape, etc. When compared to chromatogram of freshly prepared solution. The solution stored under room temperature was stable up to 32 hours and under refrigeration up to 50 hours.

ANALYSIS OF FORMULATION

After the development and validation of the method it was applied to analysis of formulation.

Recording of chromatograms

A steady baseline was recorded with the fixed chromatographic conditions, and standard drug solutions were injected and chromatograms were recorded. Retention time of bosentan and aceclofenac were found to be 6.2 and 8.5 minutes respectively. This was followed by injection of sample solution obtained from the formulation (Fig.5). The results of formulation are given in table 10.

Table 10: Analysis of formulation

Formulation	Amount of drug (mg/tablet)		% label claim	% RSD*
	Labeled	Estimated		
Lupibose (Bosentan)	62.5	62.39	99.83	0.539

**Average of six determinations*

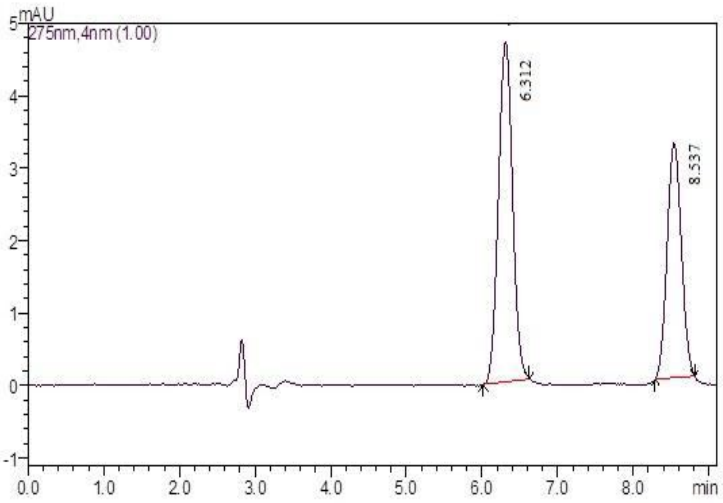

Fig. 5: Chromatogram of formulation – 2.5 µg/mL

***IN VITRO* INTERACTION STUDY OF BOSENTAN WITH ACECLOFENAC BY EQUILIBRIUM DIALYSIS METHOD**

Preparation of phosphate buffer pH 7.4: The buffer solution was prepared by weighing 3.532 gm of potassium dihydrogen phosphate and 14.542 gm of disodium hydrogen phosphate are dissolved in 1000 ml of distilled water.

Preparation of bovine serum albumin ($1.5x10^{-4}$M): The solution of bovine serum albumin was prepared by weighing 0.512 gm of bovine serum albumin, dissolved and made upto 50 ml with phosphate buffer solution of pH 7.4 in a volumetric flask.

Preparation of standard solutions: Stock solutions of bosentan and aceclofenac were separately prepared by dissolving 10mg each in 10 ml methanol. 10 ml of the above solution was diluted to 100 ml with phosphate buffer solution of pH 7.4 to get a concentration of 100µg/ml. The above stock solution of aceclofenac was diluted with phosphate buffer solution to get a series of concentrations containing 5-30 µg/ml of aceclofenac.

Optimisation of bosentan concentration and equilibrium time

Protein binding of bosentan was determined by equilibrium dialysis method. Equilibrium dialysis is one of the methods used for the determination of protein binding and this method is used to study the complexation between BSA and the drug. If binding occurs, the drug concentration in the sac containing the protein is greater at equilibrium than the concentration of drug in the vessel outside the sac. At regular intervals samples were withdrawn and analysed to obtain the concentration of free and complexed drug. The dialysis membranes (Hi media dialysis membrane- 110, 21.5 mm diameter and 20 cm length) were previously activated by immersing it in warm water at 70˚C for 1 hour. About 5 ml of $1.5x10^{-4}$M BSA solution was taken in the bag and immersed in 25ml of phosphate buffer containing varying concentrations of bosentan ($1.75x10^{-6}$ – $1.05x10^{-5}$M) and shaken gently in a mechanical shaker with an rpm of 84±1 at room temperature. Immediately at zero time, 1 ml of the solution was pipette out from the conical flask and it was replaced with 1 ml of phosphate buffer solution of pH 7.4. Samples were drawn at different intervals and replaced the same volume with phosphate buffer. Then the samples were injected into the HPLC system and peak areas were noted at 275nm. Samples were drawn until equilibrium is attained and no more increase in the areas or fall in the peak areas occur.

Interaction studies

Study of effect of aceclofenac on *in vitro* protein binding of bosentan

To study the effect of aceclofenac on *in vitro* protein binding of bosentan, 5 ml of $1.5x10^{-4}$M BSA solution was taken in each of seven dialysis bags. The dialysis membranes were previously activated by immersing it in warm water at 70˚C for 1 hour. The bags were then immersed in conical flasks with 25 ml of phosphate buffer containing fixed concentration of bosentan ($1.05x10^{-5}$M). Aceclofenac was added in increasing concentrations into six conical flasks containing bosentan solution to give a final ratio (BSA: Bosentan: Aceclofenac, 1:1:0, 1: 1: 1, 1: 1: 2, 1: 1: 3, 1: 1: 4, 1: 1: 5, 1: 1: 6) and the 7th one was used as control.

The system was shaken gently in a mechanical shaker with an rpm of 84±1 at room temperature. Immediately at zero time, 1 ml of the solution was pipette out from the conical flask and it was replaced with 1 ml of phosphate buffer solution. After 24hrs, 1 ml of solution was withdrawn and injected into the HPLC system and peak areas were noted. From the table 11 and fig. 6 it was observed that the free concentration of bosentan increased from 10.76 to 17.72% that is aceclofenac at higher concentration displaced bosentan to a greater extent, so aceclofenac has greater affinity for protein binding site on bovine serum albumin molecule than bosentan.

Table 11: Effect of aceclofenac on *in vitro* protein binding of bosentan

BSA: Bosentan:Aceclofenac	% Protein binding	% Free drug concentration
1:1:0	89.45	10.76
1:1:1	88.17	11.83
1:1:2	87.82	12.18
1:1:3	85.86	14.14
1:1:4	84.99	15.01
1:1:5	84.86	15.64
1:1:6	82.28	17.72

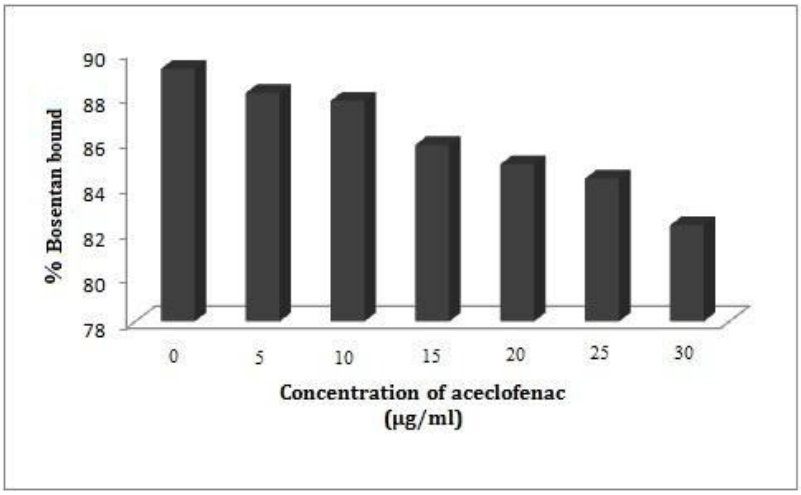

Fig 6: Effect of aceclofenac on *in vitro* protein binding of Bosentan

CONCLUSION

The results of analysis have been validated as per ICH guidelines and recovery studies confirmed the reproducibility and accuracy of the proposed RP-HPLC method. Analysis of authentic sample containing bosentan showed no interference from the common additives and excipients. The method was found to be simple, sensitive and precise. Hence, recommended procedure is well suited for the assay and evaluation of drugs in commercial tablets. The

method was also successfully applied to the *in vitro* drug interaction study of aceclofenac which is one of the commonly used NSAID. From the study it may be concluded that bosentan should not be co-administered with aceclofenac. Otherwise the concomitant therapy may increase the free fraction of bosentan and it may lead to serious liver injury. However, from our limited data it is too early to draw such conclusion about the pharmacokinetic properties of the drug. It deserves a more detailed study using *in vivo* experimental model.

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