Validated Stability Indicating High Performance Liquid Chromatographic Method for the Determination of Ambrisentan in Pharmaceutical Dosage Form

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A R T I C L E I N F O

A B S T R A C T

Background: A simple, sensitive, selective and precise stability indicating HPLC method is developed and validated for the assay of ambrisentan in pharmaceutical dosage form. ***Methods:*** Separation of the drug was achieved on Agilent Zorbax C18 column (250 mm × 4.6 mm I.D., 5 µm particle size) under the isocratic mode of elution. The solvent system consisted of 0.1M potassium dihydrogen phosphate buffer (pH was adjusted to 4.4 with orthophosphoric acid) and methanol (30:70 *v/v*) at a flow rate of 1.0 ml/min. The method was carried out in the absorbance mode at 210 nm. The method was statistically validated according to International Conference on Harmonization guideline. Ambrisentan was subjected to stress degradation studies under acidic, basic, oxidative, thermal and photolytic conditions. ***Results:*** The system was found to give compact peak for ambrisentan (Retention time is 3.315 min). The method was linear in the range of 1 - 150 µg/ml. The linear regression data for the calibration plot showed good relationship (r^2 = 0.9996). The relative standard deviation and mean recovery values at different concentration levels were within limits. The performance of the method was not changed when small variations in the experimental conditions were made. Degradation products resulting from stress degradation studies did not interfere with the detection of ambrisentan. ***Conclusions:*** The proposed stability indicting HPLC method is simple, precise, accurate, robust and selective. This method can be used for quantification of ambrisentan in bulk drug and in pharmaceutical dosage forms.

Introduction

Ambrisentan (ABN) is an orally active and highly selective endothelin A-receptor antagonist[1-4]. It is approved by the US Food and Drug Administration in 2007 for the treatment of pulmonary arterial hypertension to improve exercise capacity and delay the clinical worsening. The quantification of ABN is not official in any pharmacopoeias. There are only a few methods reported for the quantification of ABN. A HPLC method for the determination of ABN enantiomers has been reported by Douša and Gibala[5]. The assay of ABS in rat plasma was done by HPLC–positive ion electrospray tandem mass spectrometry[6]. There are few reports on the use of visible spectrophotometry in the determination of ABN[7,8]. The above reported HPLC methods are not applied for the assay of ABN in pharmaceutical dosage form. The spectrophotometric methods suffer from lack of sensitivity and requires extraction step. Therefore, the need for a simple and sensitive method is obvious for the analysis of ABN in pharmaceutical dosage form.

The aim of this study was to develop and validate a simple, rapid and sensitive stability indicating HPLC method for the determination of ABN in pharmaceutical dosage form.

Materials and methods
Chemicals and reagents
Ambrisentan was provided by MSN laboratories, Hyderabad and was used as received. Ambrisentan tablets (Letairis tablets, 10 mg/tablet, Gilead Sciences, Inc., CA, US) were obtained from commercial sources in the pharmacy market. Milli-Q-water, methanol, orthophosphoric acid and potassium dihydrogen phosphate of HPLC grade was received from Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India.

Chromatographic apparatus
During method development and validation, Waters Alliance HPLC systems equipped with 2695 separation modules having 2996 photodiode array detector was used. Data acquisition, analysis and processing were

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done using Millennium 32 software. The analytical column used for the separation was 250 mm × 4.6 mm I.D., 5 μm particle size, Agilent Zorbax C18 column.

Chromatographic Conditions

HPLC analysis was conducted in a reverse phase Agilent Zorbax C18 column. (250 mm × 4.6 mm I.D., 5 μm particle size) using isocratic elution. The column was thermostated during the analysis at 30±1°C. The mobile phase consisted of 0.1 M potassium dihydrogen phosphate buffer and methanol (30:70 *v/v*) at a flow rate of 1 ml/min. The pH of the phosphate buffer was adjusted to 4.4 with 85% orthophosphoric acid. The mobile phase was filtered through 0.45 mm membrane filter and degassed with a helium sparge for 15 minutes before use. The injection volume was 10 μL. The effluent was monitored at 210 nm.

Preparation of standard solution

In preparing the standard solution, accurately weighed 50 mg of ABN was transferred to a 50 ml volumetric flask, dissolved in and diluted up to the mark with mobile phase to obtain a stock standard solution of ABN (1 mg/ml). This solution was further diluted with mobile phase to obtain working standard solutions of ABN in concentration range of 1–150 μg/ml.

Preparation of sample solution

In preparing the sample solution, ten tablets were weighed to acquire the average weight and then powdered. The powder equivalent to 50 mg of ABN was weighed and transferred into a 50 ml volumetric flask and dissolved using mobile phase. This mixture was sonicated for 15 minutes and filtered through a 0.45 mm membrane filter. The filtered solution was aptly diluted with mobile phase to obtain a concentration of 100 μg/ml.

Preparation of placebo solution

In preparing the placebo solution, starch (10 mg), acacia (10 mg), hydroxyl cellulose (10 mg), sodium citrate (10 mg), talc (10 mg), magnesium stearate (10 mg), lactose (10 mg), glucose (10 mg) and sodium alginate (10 mg) was weighed accurately and transferred into a 100 ml volumetric flask. Approximately 50 ml of mobile phase was added to the volumetric flask, which was then sonicated for 15 minutes. The resulting solution was filtered through a 0.45 mm membrane filter and was then diluted up to the mark with mobile phase and mixed well.

Method validation

The developed HPLC method was validated by determining the following parameters: such as system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, robustness, selectivity and specificity, according to ICH guidelines[9,10].

System suitability studies

System suitability parameters, such as retention time, peak area, tailing factor, number of theoretical plates, plates per meter and height equivalent to theoretical plates were measured by means of five injections of a standard drug solution containing 100 μg/ml of ABN. Relative standard deviations of the each parameter were measured to test the system suitability.

Linearity

Linearity was determined by constructing analytical curve with ten calibration points for ABN, with the concentrations 1, 5, 10, 25, 50, 75, 100, 125 and 150 μg/ml. The peak area values were plotted against the respective concentrations of ABN to get the analytical curve. The results were subjected to regression analysis by the least squares method to calculate the slope, intercept and regression coefficient.

LOD and LOQ

The sensitivity of the method was expressed in terms of limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were determined by using the formulae:

LOD= 3 x standard deviation of peak area/slope of the calibration curve
LOQ= 10 x standard deviation of peak area/slope of the calibration curve

For this purpose, five replicate injections of dilute solutions with known concentration (1 μg/ml) were injected into the HPLC system and peak area was recorded.

Precision and accuracy

Precision and accuracy was determined by five replicate analyses of each of the concentrations of 1 μg/ml, 75 μg/ml, 150 μg/ml of standard ABN solutions using the proposed method on the same day (intra-day precision and accuracy) and for three consecutive days (inter-day precision and accuracy). The relative standard deviation and percent recovery was then calculated to represent precision and accuracy, respectively.

Accuracy of the method was further confirmed by recovery studies through standard addition method. In the standard addition method known quantities of ABN at three different concentration levels (50, 100 and 150% of the labeled claim) were supplemented to the tablet sample solution previously analyzed. The solutions were once again analyzed by the proposed method. The percentage recoveries and relative standard deviations were calculated.

Robustness

Robustness of the method was assessed by varying the parameters like mobile phase ratio, buffer pH, flow rate

and temperature deliberately. In these experiments, one parameter was changed while the others were kept unchanged. The relative standard deviation for the peak area was calculated each time. The assay was carried out at two different concentration levels of ABN (1 and 150 µg/ml).

Selectivity

The selectivity of the method was assessed by evaluating the interference of the excipients of the pharmaceutical formulation and components of the mobile phase. For this, chromatograms of standard solution with the ABN concentration of 100 µg/ml, mobile phase blank, placebo and tablet samples were compared.

Specificity (forced degradation studies)

The forced degradation studies were performed to show whether the proposed method was stability-indicating and could evaluate the analyte in the presence of degradation products. The ABN was stressed under acid hydrolytic, alkali hydrolytic, oxidative, thermolytic and photolytic stress conditions.

Alkali hydrolysis

Accurately weighed 100 mg of ABN was transferred into a 100 ml volumetric flask and 10 ml of 0.1 N NaOH was added. The flask was heated on a water bath at 80ºC for 2 hours. It was allowed to cool, neutralized with 0.1 N HCl and the volume was made up to the mark with mobile phase. Suitable aliquot was taken from the solution and diluted with mobile phase to get final concentration of 100 µg/ml of ABN.

Acid hydrolysis

Accurately weighed 100 mg of ABN was transferred into a 100 ml volumetric flask and 10 ml of 0.1 N HCl was added. The flask was heated on a water bath at 80ºC for 2 hours and allowed to cool. The solution was neutralized with 0.1 N NaOH. The volume was made up to the mark with mobile phase. Appropriate aliquot was taken from the solution and diluted with mobile phase to obtain final concentration of 100 µg/ml of ABN.

Oxidative stress degradation

To perform oxidative stress degradation, 100 mg of ABN was accurately transferred into a 100 ml volumetric flask. Ten ml of 3% hydrogen peroxide was added to it. The mixture was heated in a water bath at 80ºC for 2 hours and allowed to cool. The volume was made up to the mark with mobile phase. The solution was suitably diluted with mobile phase to obtain final concentration of 100 µg/ml of ABN.

Dry heat degradation

Hundred mg of ABN was exposed to dry heat at 105ºC for 2 hours in a hot air oven. The sample was allowed to cool, transferred to a 100 ml volumetric flask and

dissolved in 30 ml of mobile phase. The volume was made up to the mark with the mobile phase. The solution was further diluted by mobile phase to obtain final concentration of 100 µg/ml of ABN.

Photolytic degradation

Hundred mg of ABN was exposed to sunlight for 24 hours. The sample was allowed to cool, transferred to a 100 ml volumetric flask and dissolved in 30 ml of mobile phase. The volume was made up to the mark with the mobile phase. The solution was further diluted with the mobile phase to get final concentration of 100 µg/ml of ABN.

All the above solutions were injected in the liquid chromatographic system and chromatograms were recorded. Assay study was performed by the comparison with the peak area of ABN sample (100 µg/ml) without degradation.

Analysis of ABN in pharmaceutical dosage form

For this purpose 10 µL of the sample solution, prepared as described under "Preparation of sample solution", was injected into HPLC system in triplicate. The chromatograms were recorded. The area under the peak was calculated. The concentration of ABN in the pharmaceutical dosage form was calculated using the analytical curve or regression equation.

Results and Discussion
Optimization of the HPLC conditions

The HPLC conditions (analytical column, column temperature, mobile phase and their proportions, pH of the buffer, flow rate and detection wavelength) were optimized with an aim to achieve good separation of ABN in presence of stress degradation products within the possible shortest time. The HPLC conditions were optimized based on parameters like retention time, tailing factor, peak height and peak area.

Various HPLC analytical columns like thermo BDS hypersil-C8 (250 mm x 4.6 mm x 5 µm), XTerra RP-C8 (150 mm x 4.6 mm x 5 µm) and Agilent Zorbax –C18 (250 mm x 4.6 mm x 5 µm) were used during method development. Based on the above said parameters Agilent Zorbax –C18 (250 mm x 4.6mm x 5 µm) column was finalized for analysis. The column maintained at a temperature of 30°C was the best value found for all parameters. For this reason, 30°C was selected as the final temperature for the analysis.

Various buffers like potassium dihydrogen orthophosphate, sodium dihydrogen orthophosphate and ammonium acetate with pH ranging from 3.0 to 9 were tried for the development. It was observed that potassium dihydrogen orthophosphate with pH of about 4.4 good peak shape, less retention time and less tailing factor were obtained. Therefore, potassium dihydrogen phosphate buffer (pH 4.4) was selected for the analysis. Organic modifiers like acetonitrile and methanol were evaluated. Satisfactory chromatographic separation was achieved when methanol was used. Hence, methanol

was finalized as organic modifier for analysis.

Mobile phase consisting of potassium dihydrogen phosphate buffer (pH 4.4) with methanol in different volumetric ratios were investigated to meet the necessary system parameters. The experiments indicated that better sensitivity was achieved while using potassium dihydrogen phosphate buffer (pH 4.4) and methanol in the ratio of 30:70 *v/v*. The mobile phase with different flow rates (0.5-1.5 ml/min) was investigated. At the flow rate 1 ml/min, symmetric and well retained peak was obtained. Therefore, for the present study the flow rate   1 ml/min was preferred.

To determine the suitable wavelength for determination of ABN, UV spectra of the ABN in mobile phase were scanned in the range 200–400. The maximum absorption wavelength for ABN was 210 nm. So, 210 nm was chosen as the detection wavelength.

Method validation

System suitability studies

The relative standard deviation values calculated for the retention time, peak area, tailing factor, theoretical plates, plates per meter and height equivalent to theoretical plates were   0.033 %, 0.521 %, 0.507 %, 1.224 %, 1.224 %, and 1.215 %, respectively. The results show that the parameters tested were within the acceptable range (RSD < 1.5 %), indicating that the system was appropriate for the proposed analysis.

Linearity

The analytical curve for ABN was constructed by plotting the peak area values versus concentration. The method was linear in the 1–150 μg/ml range with a regression coefficient of 0.9996. The linear regression equation was A = 20374 x + 14080 (where A = peak area and   x = concentration of ABN in μg/ml). The

high regression coefficient value was suggestive for the good linearity of the proposed method.

LOD and LOQ

The calculated LOD and LOQ values were 0.244 and 0.812 μg/ml, respectively. The low values of LOD and LOQ suggest the high detection ability of the method.

Precision and accuracy

The results of intra-day and inter-day precision and accuracy are shown in table 1.   The low relative standard deviation (<1), excellent recovery and error values lower than 0.5 % proved that the method is adequately precise and accurate.

The results obtained for the recovery study are presented in table 2. The mean recovery obtained for each level, as well as for all levels showed that the method was accurate and suitable for the determination of ABN.

Robustness

The results of the method robustness are summarized in table 3. The low relative standard deviation (<0.5) indicated that small variation in the experimental variables did not significantly affect the analytical performance of the method.

Selectivity

The chromatograms of standard ABN solution, mobile phase blank, placebo and tablet samples are represented in Figure 1. There is no peak interference of blank and placebo at the retention time of ABN. The results indicated that there is no drug-excipient interaction. Therefore, the method is selective for the determination of ABN in their pharmaceutical dosage form.

Table 1. Results of precision and accuracy of the method

Analysis	Concentration of ABN (μg/mL)		% RSD	% Recovery	% Error
	Taken	Found[a] ± SD			
Intra-day	1	0.998 ± 0.003286	0.329	99.800	0.200
	75	75.007 ± 0.075919	0.101	100.009	0.009
	150	150.165 ± 0.104648	0.069	100.110	0.110
Inter-day	1	0.997 ± 0.003786	0.380	99.700	0.300
	75	74.946 ± 0.066144	0.088	99.928	0.072
	150	150.380 ± 0.095824	0.063	100.253	0.253

[a] average of five determinations

Table 2. Results of recovery studies of the method

ABN in tablet (mg)	Pure ABN added (mg)	Total found[a] (mg) ± SD	% RSD	% Recovery
10	5	14.891 ± 0.046508	0.312	99.293
10	10	19.922 ± 0.017010	0.085	99.610
10	15	24.871 ± 0.020526	0.082	99.484

[a] average of five determinations

Table 3. Results of robustness of the method

Parameter	Concentration of ABN (µg/mL)	Peak area[e]	% RSD
Mobile phase ratio[a]	1	21841	0.403
	150	3243229	0.115
Buffer pH[b]	1	21708	0.294
	150	3242431	0.046
Flow rate[c]	1	21641	0.240
	150	3245320	0.013
Temperature[d]	1	21701	0.032
	150	3242726	0.016

[a] Phosphate buffer and methanol ratios (*v/v*)- 28:72, 30:70 and 32:68
[b] Buffer pH – 4.3, 4.4 and 4.5
[c] Flow rate (mL/min) – 0.9, 1.0 and 1.1
[d] Temperature (^{o}C) – 28, 30 and 32
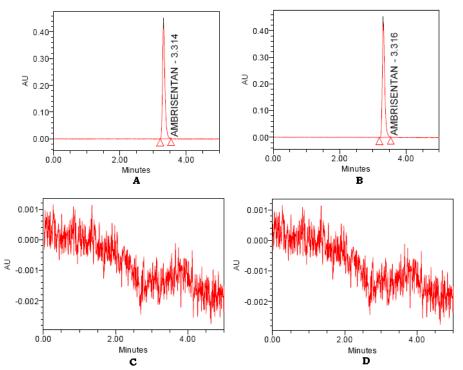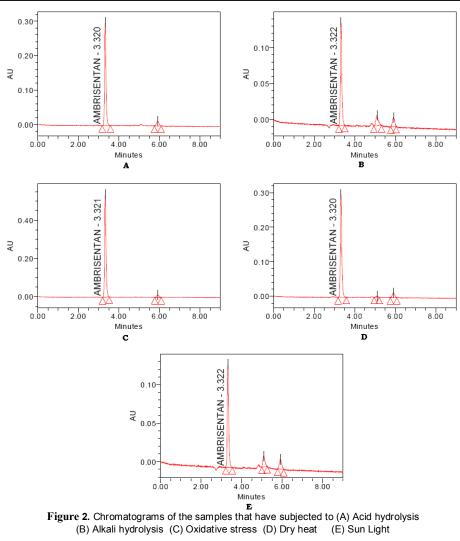[e] Mean of three values

Figure 1. Chromatogram of   (A) Pure sample (B) Tablet sample (C) Placebo (D) Blank mobile phase

Specificity

The specificity of the proposed method was analyzed at 100 µg/ml concentration. The study was made based on the peak area of the respective sample. Less degradation occurred under oxidative stress condition with percent decomposition being only 3.053% and more degradation occurred under photolytic degradation (51.064%). The percentage of ABN degradation under acid, alkaline and dry heat conditions was found to be 4.126%, 16.749% and 13.355%, respectively. The results are summarized in table 4. The chromatograms obtained for ABN after subjecting to degradation are presented in Figure 2. In all the cases, the proposed HPLC method was able to separate completely the degradation products from the intact ABN. This confirmed the specificity and stability-indicating property of the proposed HPLC method.
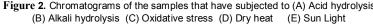
Figure 2. Chromatograms of the samples that have subjected to (A) Acid hydrolysis (B) Alkali hydrolysis (C) Oxidative stress (D) Dry heat (E) Sun Light

Table 4. Results of forced degradation studies

Type of stress	Peak area	% Recovery	% degradation
Undegraded	2167268	-	-
Acid hydrolysis	2077848	95.874	4.126
Alkali hydrolysis	1804283	83.251	16.749
Oxidative stress	2101102	96.947	3.053
Dry heat	1060586	48.936	51.064
Photolytic	1877848	86.645	13.355

Table 5. Results of Assay of ABN in tablets

ABN in Tablet (mg)	ABN Found (mg)	
10	9.986	Mean: 9.992 mg
10	9.985	% RSD: 0.118
10	10.006	% Recovery: 99.920

Application of the method
The developed method was applied to the determination of ABN in pharmaceutical dosage forms. Satisfactory results were obtained for ABN and were found to be in agreement with the label claims (Table 5). The analysis of tablet dosage form indicated that the method is selective for determination of ABN in the pharmaceutical dosage form.

Comparison of the proposed method with the reported liquid chromatographic methods
In comparison with the earlier reported liquid chromatographic methods[5,6] for the analysis of ABN, the proposed method is economical, specific, accurate, precise and applicable to commercial tablet dosage forms. The method reported by Dousa and Gibala[5] has a very narrow range of linearity (0.15 μg/ml to 3 μg/ml), less precise and less accurate with RSD values and percent error in the range of 1.6-2.0% and 3.4%, respectively. From the results of intra- and inter-day precision and accuracy (Table 1), it was seen that the RSD and percent error of the proposed method is in the range of 0.063 – 0.329 % and 0.009-0.30% respectively. In the Dousa and Gibala method[5], the use of acetonitrile in the mobile phase in turn increases the cost of the analysis. Unlike the Nirogi et al.[6] method, the proposed method does not require extraction procedure, internal standard and costly detector system. These factors increase the simplicity of the proposed method and decrease the cost of the analysis. In both the reported methods[5,6], forced degradation studies were not reported and the methods[5,6] were not applied to commercial tablets formulations.

Conclusion
The present study deals with the development of a stability-indicating HPLC method for quantification of ABN in bulk and pharmaceutical dosage forms. The developed method was validated following the recommendations of ICH guidelines. The results indicated that the method presented acceptable precision, accuracy, selectivity and wide linear concentration range. Results of specificity proved that the method is suitable for the determination of ABN with no interference from the degradation products. The developed method is capable for quantitative analysis of ABN in the pharmaceutical dosage form.

Acknowledgements

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