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A validated LC method for the enantiomeric separation of ambrisentan in bulk drug and pharmaceutical dosage forms

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ABSTRACT

A new and accurate chiral liquid chromatographic method has been developed for the separation of Ambrisentan and its (R)-enantiomer in bulk drugs and pharmaceutical dosage forms. Normal phase chromatographic separation was performed on an immobilized cellulose based chiral stationary phase (Chiralpak-ADH) with n-hexane: ethanol (85:15, v/v) as mobile phase at a flow rate of 1.0 mL min^{-1}. The elution time was approximately 15 min. The resolution (R_s) between the enantiomers was greater than 3.0. The limit of detection (LOD) and limit of quantification (LOQ) for the (R)-enantiomer were 0.03 μg and 0.1 μg respectively, for a 10 μL injection volume. The linearity of the method for the (R)-enantiomer was excellent (r^2 >0.999) over the range from LOQ to 0.3%. Percentage recovery of the (R)-enantiomer from bulk drug samples and pharmaceutical dosage forms ranged from 98.5-101.2% indicative of the high accuracy of the method. Robustness studies were also conducted. The sample solution stability and mobile phase stability studies were determined and the results were found to be satisfactory for a study period of 48 h.

KEYWORDS

Ambrisentan;
Chiral HPLC;
Enantiomeric separation;
Validation;
Quantification.

INTRODUCTION

Ambrisentan is a cardio vascular drug and it known as potent type-A selective endothelin receptor antagonist which has been proposed for the treatment of pulmonary arterial hypertension (PAH). The chemical name of Ambrisentan is (+)-(2S)-2-[(4,6-dimethylpyrimidin-2-yl)oxy]-3-methoxy-3,3-diphenylpropanoic acid (Figure 1). Endothelin is a small peptide hormone that is believed to play a critical role in control of blood flow and cell growth. Endothelin receptor antagonists (ETRAs) are a class of drugs which prevent the constriction or narrowing of blood vessels thereby enhancing blood flow throughout the body. There are two classes of endothelin receptors: Endothelin A (ET-A) and Endothelin B (ET-B). The binding of endothelin to ET-A receptors causes vasoconstriction while the binding to ET-B causes vasodilatation. Ambrisentan is a high affinity ET-A receptor antagonist with a high selectivity for the ET-A versus ET-B receptor. Elevated endothelin blood levels are associated with several cardiovascular disease conditions, including not only PAH but also chronic renal disease, coronary artery disease, hypertension and chronic heart failure.

Pulmonary arterial hypertension (PAH) is a disease characterized by excessive constriction of the blood ves-

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sels in the lungs. Such constriction leads to high pulmonary arterial pressures, making it difficult for the heart to pump blood through the lungs. Since blood is oxygenated in the lungs, this constriction prevents the lungs from adequately oxygenating the blood, which causes persons afflicted with pulmonary arterial hypertension to suffer from extreme shortness of breath as the heart struggles to oxygenate the blood by pumping against high arterial pressures[1-11].

Enantiomers of racemic drugs often differ in pharmacokinetic behaviour or pharmacological action[12]. The development of analytical methods for the quantitative analysis of chiral materials and for the assessment of enantiomeric purity is extremely challenging due to the fact that enantiomers posses virtually identical properties[13]. Although many analytical techniques like gas chromatography (GC), capillary electrophoresis (CE), liquid chromatography (LC) can be employed to achieve this, the most widely used is liquid chromatography (LC) employing a chiral stationary phase (CSP)[14-16].

So far, to our present knowledge no chiral HPLC methods were reported in the literature for the enantiomeric separation of Ambrisentan and accurate quantification of its enantiomeric impurity namely (R)-enantiomer in bulk drugs and pharmaceutical dosage forms.

As the active ingredient in Letairis tablets was (S)-Ambrisentan. It is felt necessary to develop a chiral LC method for the enantiomeric separation and accurate quantification of unwanted enantiomer ((R)-enantiomer) of Ambrisentan. This paper deals with method development and validation for the enantiomeric separation of Ambrisentan and accurate quantification of its enantiomeric impurity namely (R)-enantiomer in bulk drugs and pharmaceutical dosage forms.

EXPERIMENTAL

Chemicals

Samples of Ambrisentan, its (*R*)-enantiomer were obtained from Research and Development Laboratory of United States Pharmacopeia-India (P) Ltd, India; the chemical structures were given in (Figure 1). Commercially available 10 mg Ambrisentan tablets (Letairis®) were purchased from Glaxo Pharmaceuticals, Mumbai, India. HPLC grade n-hexane and ethanol was purchased from Merck, Darmstadt, Germany.

Equipment

Method development and validation were performed with Waters (Waters Corporation, Massachusetts, USA) Alliance equipment comprising a model 2695 separation module with inbuilt auto injector and a model 2996 photo diode array detector. The output signal was monitored and processed by use of Empower software on a Pentium computer (Digital equipment Co.). The Photo diode array detector was also used for determination of peak purity.

Chromatographic conditions

The chromatographic conditions were optimized using the Chiralpak-ADH column of Daicel make (Daicel Chemical Industries Ltd., Japan) with 5 μm particle size in (250 × 4.6) mm dimension. The mobile phase was *n*-hexane: ethanol (85:15, v/v) at a flow rate of 1.0 mL min^{-1}. The column temperature was maintained at 25^0C and the detection was at 220 nm. The injection volume was 10 μL. Mobile phase was used as diluent.

Preparation of standard solutions

Individual stock solutions of Ambrisentan, its (R)-enantiomer (1000μg mL^{-1}) were prepared by dissolving appropriate amounts of the substances in the diluent. Working solutions of Ambrisentan, its (R)-enantiomer were also prepared in diluent.

Preparation of sample solution

Letairis® tablets contain 10 mg Ambrisentan. The inactive ingredients present in Letairis are croscarmellose sodium, lactose monohydrate, magnesium stearate and microcrystalline cellulose. The tablets are film-coated with a coating material containing FD and C Red #40

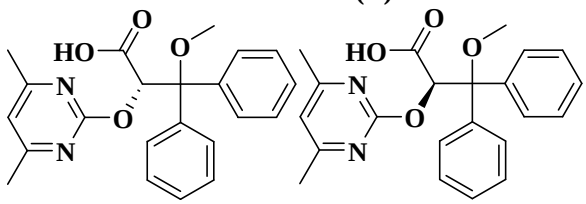

Figure 1: Structures and labels of Ambrisentan and (R)-Ambrisentan.

Aluminum Lake, lecithin, polyethylene glycol, polyvinyl alcohol, talc, and titanium dioxide. Twenty Letairis tablets (10 mg) were weighed and the average weight was calculated. The tablets were then powdered in a mortar and a sample of the powder equivalent to 50 mg of the active pharmaceutical ingredient (Ambrisentan) was transferred to 100 mL volumetric flask. Approximately 75 mL diluent was added and the flask was placed on rotatory shaker for 10 min and sonicated for 10 min to dissolve the material completely. The solution was then diluted to 100 mL and centrifuged at 3,000 rpm for 10 min. The supernatant was collected and filtered through a 0.45 μm pore size Nylon 66-membrane filter. The filtrate was used as sample solution.

Method validation

The method was validated in accordance with ICH guidelines[17-19].

Precision

The precision of the method was carried out at four different concentration levels by injecting six replicate preparations of 500 μg mL^{-1} Ambrisentan sample spiked with LOQ, 0.075%, 0.15% and 0.225% of its (R)-enantiomer on the same day was calculated to determine intra-day precision. This study was also evaluated for 3 days to determine inter-day precision.

The intermediate precision of the method was also evaluated on a column from different batch, on a different instrument, by a different analyst on the same instrument, and in different laboratories. %RSD for peak area of (R)-enantiomer was calculated.

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

The LOD, defined as lowest concentration of analyte that can be clearly detected above the baseline signal, is estimated as three times the signal to noise ratio. The LOQ, defined as lowest concentration of analyte that can be quantified with suitable precision and accuracy, is estimated as ten times the signal to noise ratio. LOD and LOQ were achieved by injecting a series of dilute solutions of (R)-enantiomer.

The precision of the developed method at LOQ was checked by analyzing six test solutions of (R)-enantiomer prepared at LOQ level and calculating the percentage relative standard deviation of peak area.

Linearity and range

Linearity for the (R)-enantiomer was evaluated by analysis of seven working sample solutions ranging from the LOQ to 200% of the permitted maximum level of impurity (0.15%). (LOQ, 0.0375 %, 0.075 %, 0.1125%, 0.15 %, 0.225 % and 0.30 %).

Peak areas and concentrations of the (R)-enantiomer were subjected to regression analysis to calculate calibration equation, correlation coefficient and Range. Linearity was checked for three consecutive days in the same concentration range from the same stock solution. The percentage relative standard deviation of the slope and Y-intercept of the calibration curve was calculated.

Accuracy

Standard addition and recovery experiments were conducted to determine accuracy of the method for quantification of the (R)-enantiomer in bulk drugs samples and pharmaceutical dosage forms of Ambrisentan.

Recovery was measured in triplicate for 0.075%, 0.15% and 0.225% of the (R)-enantiomer relative to the Ambrisentan target analyte concentration (500 μg mL^{-1}). The percentage recovery of (R)-enantiomer was calculated.

Robustness

A method is robust if results are not affected by small but deliberate variation of the method conditions. This was studied to anticipate problems which may arise during regular application of the method. To assess robustness the experimental conditions were altered and resolution between Ambrisentan and its (R)-enantiomer was checked. To study the effect of flow rate, the flow was changed from 1.0 to 0.9 and 1.1 mL min^{-1}. The effect of column temperature on resolution was studied at 23^0C and 27^0C instead of 25^0C.

The effect of mobile phase composition on resolution was assessed by changing the amounts of ethanol by ±1%. When any condition was varied, the other conditions were held constant.

Solution stability and mobile phase stability

The solution stability for Ambrisentan and its (R)-enantiomer was assessed by leaving both unspiked and

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spiked sample solutions in tightly capped volumetric flask at room temperature on a laboratory bench for 48 h. The amount of the (R)-enantiomer was determined at 6 h intervals and compared with that in freshly prepared solution at each time point.

TABLE 1: System suitability report

Name	Retention time(t_r) in min	USP Resolution (R_S)	USP Tailing factor (T)	Theoretical plates(N)
Ambrisentan	7.6	-	1.09	4216
(R)-Ambrisentan	9.3	3.5	1.02	4102

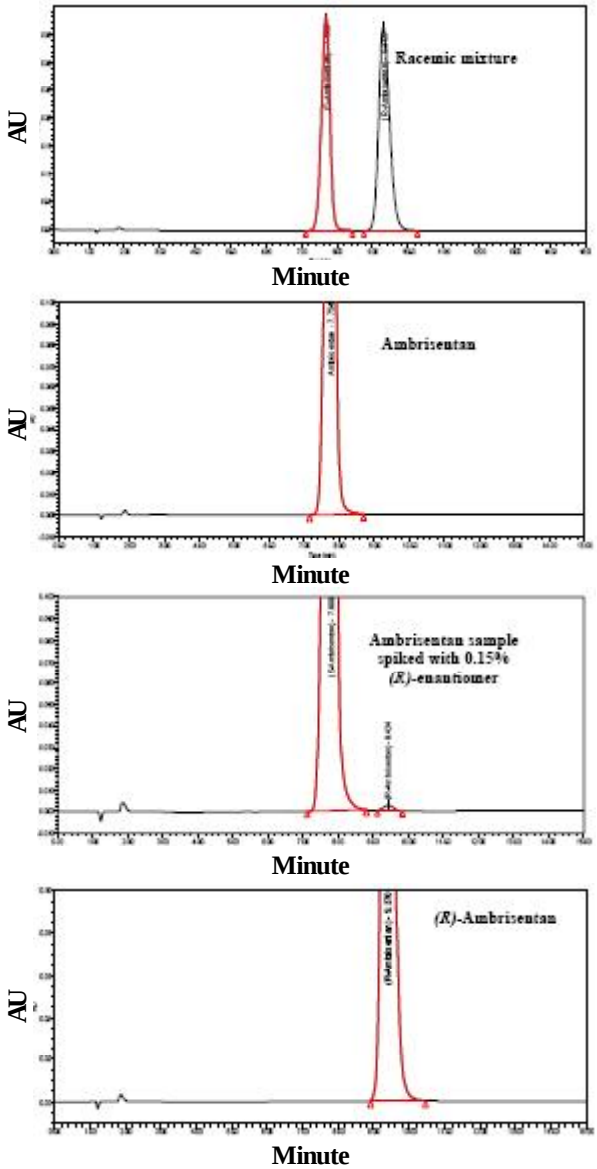

Figure 2: Typical chromatogram of racemic mixture, Ambrisentan sample, Ambrisentan sample spiked with (R)-enantiomer at 0.15% level, (R)-enantiomer of Ambrisentan

Mobile phase stability was also carried out for 48 h by injecting the freshly prepared sample solutions for every 6 h interval. Content of (R)-enantiomer of Ambrisentan was checked in the test solutions. Mobile phase prepared was kept constant during the study period.

RESULTS AND DISCUSSION

Method development and optimization

The objective of this study was separation of Ambrisentan enantiomer and accurate quantification of (R)-enantiomer of Ambrisentan in bulk drugs and pharmaceutical dosage forms. A mixture of Ambrisentan and its (R)-enantiomer were used during the method development. Preliminary trial was performed with polysaccharide type chiral column namely Chiralpak AD-H. Poor separation was observed with Chiralpak AD-H when n-hexane: isopropyl alcohol (80:20, v/v) was used as mobile phase. Replacement of isopropyl alcohol with ethanol improved the resolution (R_s~2.1). Further trials were continued by changing the composition of the solvents in the mobile phase. Finally, the better chromatographic results were noticed with n-hexane: ethanol (85:15, v/v). In the optimized method the typical retention times of Ambrisentan and its (R)-enantiomer were about 9.6 and 10.9 min, respectively, and the resolution between the enantiomers was found to be greater than 3.0.

The enantiomeric separation of Ambrisentan on Chiralpak AD-H column was shown in (Figure 2). The USP tailing factor for Ambrisentan and its (R)-enantiomer was about 1.0 in the developed method. The system suitability test results are presented in TABLE 1. Mobile phase was used as blank; there was no interference of blank with Ambrisentan and (R)-enantiomer. The interference of excipients (croscarmellose sodium, lactose monohydrate, magnesium stearate and microcrystalline cellulose) was also checked by injecting sample solutions of excipients. There was no interference of excipients with Ambrisentan and (R)-enantiomer peaks.

Validation results of the method

Precision

For intra-day precision study, the %RSD value was

TABLE 2: Precision study

Validation parameter	Results (%RSD)			
	LOQ[a]	0.075%[a]	0.15%[a]	0.225%[a]
Intra-day precision (n = 6) Area ((R)-enantiomer)	2.8	2.9	2.4	2.7
Inter-day precision (n = 18) Area ((R)-enantiomer)	2.9	2.7	2.1	2.5

n=Number of preparations, [a] =Ambrisentan sample spiked with (R)-enantiomer at different concentration levels

TABLE 3: Recovery results of (R)-enantiomer in bulk drug sample

Added (µg) (n= 3)	Recovered (µg)	% Recovery	% R.S.D
0.375	0.372	99.2	0.4
0.75	0.754	100.5	0.3
1.125	1.138	101.2	0.6

n, Number of determinations

TABLE 4: Recovery results of (R)-enantiomer in dosage forms

Added (µg) (n= 3)	Recovered (µg)	% Recovery	% R.S.D
0.375	0.369	98.5	0.3
0.75	0.758	101.1	0.5
1.125	1.133	100.7	0.7

n, Number of determinations

TABLE 5: Results of robustness study

S.no.	Parameter	Variation	Resolution between Ambrisentan and (R)-enantiomer
1	Temperature	(a) At 23°C	3.3
		(b) At 27°C	3.5
2	Flow rate	(a)At 0.9 mL min^{-1}	3.5
		(b)At 1.1 mL min^{-1}	3.4
3	Ethanol	(a) At -1%	3.4
		(b) At +1%	3.5

within 3.0 % for (R)-enantiomer peak area. For inter-day precision study, the values were in the same order of magnitude than those obtained for intra-day precision confirming the high precision of the method (TABLE 2).

For intermediate precision study, the %RSD value was within 3.5% for (R)-enantiomer peak area confirming the ruggedness of the method.

Limit of detection and limit of quantification

The LOD for (R)-enantiomer of Ambrisentan was 0.03 µg (in an Ambrisentan concentration of 500 µg mL^{-1}) for a 10 µL injection volume. Under the same conditions the LOQ was 0.1 µg. The precision at LOQ concentration was below 3.0 % RSD.

Linearity and range

Good linearity (correlation coefficient r^2>0.999) was observed for (R)-enantiomer of Ambrisentan over the concentration ranges tested; the linear regression equation was y=140246*x*+82. Linearity was checked for the (R)-enantiomer over the same concentration ranges on three consecutive days. %RSD of the slope and *Y*-intercept of the calibration plot for (R)-enantiomer was 3.7 and 5, respectively. These results are indicative of excellent correlation existed between the peak area and concentration.

The Range of the method for (R)-enantiomer was found from 0.02% (LOQ) to 0.225% of the analyte concentration (500 µg mL^{-1}).

Accuracy

Ambrisentan bulk samples showed the presence of 0.03% of (R)-enantiomer. Recovery of the (R)-enantiomer from bulk drug samples of Ambrisentan was ranged from 98.2 to 101.1% (TABLE 3).

Ambrisentan dosage forms showed the presence of 0.04% of (R)-enantiomer. Recovery of the (R)-enantiomer from Ambrisentan dosage form was from 98.6 to 101.3% (TABLE 4).

Robustness

When the chromatographic conditions flow rate, temperature and mobile phase composition were deliberately varied, resolution between the Ambrisentan and its (R)-enantiomer was always greater than 3.0, illustrating the robustness of the method (TABLE 5).

Solution stability and mobile phase stability

No significant changes were observed in the (R)-enantiomer content of Ambrisentan sample during solution stability and mobile phase stability experiments when performed using the developed method. The solution stability and mobile phase stability experiments data confirms that sample solutions and mobile phase used during the study were stable up to 48 h.

CONCLUSIONS

A new and accurate normal phase chiral LC method has been established for the enantiomeric separation of Ambrisentan. Chiralpak-AD was found to be selective

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for the enantiomers of Ambrisentan. The method was validated and satisfactory results were obtained from all the method validation parameters tested. The method enables robust separation and quantification of chiral impurity ((R)-enantiomer) in bulk drug samples and pharmaceutical dosage forms of Ambrisentan.

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