SIMPLE VALIDATED RP-HPLC METHOD FOR ESTIMATION OF AMBRISENTAN IN PHARMACEUTICAL DOSAGE FORMS

RAMADOSS KARTHIKEYAN[1], ORUGANTI SAI KOUSHIK[2], P. SRINIVASA BABU[3]

ABSTRACT

The aim of the present work was to develop and validate a simple, economical, efficient method for the analysis of Ambrisentan in pharmaceutical dosage forms by reverse phase high pressure liquid chromatography. A WELCHROM C-18 reverse phase column (4.6 x 250 mm, 5 µm). With mobile phase containing 10mM Phosphate Buffer (pH 6.0): Acetonitrile (50:50, v/v) is used and eluents were monitored at 226nm. The retention time of Ambrisentan was 2.9min. The method showed a good linearity in the concentration range of 6-30µg/mL. The validation characteristics included specificity, linearity and limit of detection, limit of quantification, precision, robustness and stability. Validation acceptance criteria were met in all cases. The percent recoveries ranged between 99.9 - 100, RSD 0.184%. The method could be successfully used for the analysis of Ambrisentan in pharmaceutical dosage forms

KEYWORDS

Ambrisentan, RP-HPLC method , Validation.

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Conflict of interest

No authors are showing conflict of interest for publishing this research work.

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INTRODUCTION

Anti-hypertensive agents hold a major share of drug market as hypertension is a major cause of health problems. The estimated market share of anti-hypertensive agents is $30 billion by 2016. As a consequence, the chances of adulteration increases due to increased market needs. Adulteration in any form is not acceptable for any drugs, especially for Anti-hypertensive agents. The reason being, when the anti-hypertensives are given in fewer amounts than prescribed causes increased Hypertension, which leads to Nephropathy, Occulopathy and damage to central nervous system.Ambrisentan (AMB), a non-peptide, is a highly selective endothelin-1 type A receptor antagonist. [1-3] AMB belongs to the antihypertensive class of drugs and is used in the treatment of pulmonary atrial hypertension in patients with WHO class II or III symptoms. Endothelin is a peptide that constricts blood vessels and elevates blood pressure. AMB blocks the effects of endothelin-1 and thus decreases blood pressure in the lungs. The thickening of blood vessels in the lungs and heart is also inhibited by AMB. AMB is chemically known as (2*S*)-2-[(4, 6-dimethylpyrimidin-2-yl) oxy]-3-methoxy-3, 3-diphenyl-propanoic acid. Most Anti-hypertensives are potent and hence requires sensitive methods for its estimation in pharmaceutical formulations or in bulk forms. Hence properly developed and validated analytical methods are necessary for quality control of the drugs in market. [4-6]

MATERIAL AND METHODS

Chemicals

Ambrisentan was provided by Hetero Drugs Limited, Hyderabad, India. All the chemicals were analytical grade: potassium dihydrogen orthophosphate and phosphoric acid from S.D. Fine-Chem Ltd., Mumbai, India, while acetonitrile (HPLC grade) and triethylamine (HPLC grade) from Merck Pharmaceuticals Private Limited (Mumbai, India). Commercial tablets of Ambrisentan were purchased from local market.

Equipment

Quantitative HPLC was performed on a high pressure gradient high performance liquid chromatograph (Shimadzu LC-20AT prominence liquid chromatograph) with two LC- 20AT VP pumps, manual injector with loop volume of 20 µL (Rheodyne), programmable variable wavelength Shimadzu SPD-20A prominence UV-Vis detector and WELCHROM C-18 Column (4.6 x 250 mm, 5 µm). The HPLC system was equipped with "Spincotech" software. Standards and chemicals used.

Preparation of Reagents and Standards

Preparation of 10 M Phosphate Buffer Solution (PH 6.0)

13.8g of potassium dihydrogen orthophosphate was dissolved in 877 ml of HPLC grade water. To this 123 mL of 0.1 M phosphoric acid was added and pH was adjusted to 6.0 with triethylamine.Then it was degassed in ultrasonicator and then filtered through 0.45 µ poresize membrane filter.

Preparation of Mobile Phase

The above prepared buffer and Acetonitrile were mixed in the proportion of 50:50 v/v and was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

Preparation of Standard Solution of Ambrisentan

About 100 mg of pure Ambrisentan was accurately weighed and dissolved in 100 mL of mobile phase to get 1mg.mL^{-1} stock solution. Working standard solution of Ambrisentan was prepared with mobile phase. The final volume was made with the mobile phase. The standard solution was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

Preparation of Tadalafil

About 100 mg of pure Tadalafil was accurately weighed and dissolved in 100 mL of mobile phase to get 1mg.mL^{-1} stock solution. Working standard solution of Tadalafil was prepared with mobile phase. The final volume was made with the mobile phase. The standard solution was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

Preparation of Sample Solution of Ambrisentan

The content of 20 tablets of Ambrisentan (Letairis 10mg) were accurately weighed and transferred into a mortar and ground to a fine powder. From this, tablet powder which is equivalent to 100 mg of Ambrisentan was taken into 100ml volumetric flask and the drug was made to dissolve with mobile phase and made up to the mark with mobile phase. The resulting solution was filtered using Whatmann Grade No.1filter paper and degassed by sonication. This solution was further suitably diluted for chromatography. [7-9]

Selection of Detector Wavelength

The UV spectra of various diluted solutions of Ambrisentan in mobile phase were recorded using UV spectrophotometer. The peak of maximum absorbance was observed at 226 nm. This wavelength was used for detection of Ambrisentan.

Preparation of Calibration Standards

About 100 mg of pure Ambrisentan was accurately weighed and dissolved in 100 mL of mobile phase to get 1 mg/mL stock solution. Working standard solution of Ambrisentan was prepared with mobile phase. To a series of 10 mL volumetric flasks, standard solutions of Ambrisentan in the concentration range of 6, 12, 18, 24, and 30 µg/mL were transferred and 6 µg of standard solution of Tadalafil was added to each flask. The final volume was made with the mobile phase.

Recommended Procedure for Assay of Ambrisentan in Tablets

The test solutions were injected into the system by filling a 20 µL fixed volume loop manual injector. The chromatographic run time of 6 min. was maintained for the elution of the drug from the column. The elutes were monitored with UV detector at 226 nm. A 20µL volume of standard and sample solutions were separately injected on HPLC system. From the peak area of Ambrisentan the amount of drug in the sample were computed. The content was calculated as an average of six determinations. [10-12]

Validation of the Proposed Method for Ambrisentan

The developed method of analysis was validated as per the ICH for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness, limit of detection (LOD) and limit of quantitation (LOQ).

System Suitability

System suitability test is an integral part of chromatographic method which would be used to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug solution at the concentration level 10 µg/mL for Ambrisentan to check the reproducibility of the system.At first the HPLC system was stabilized for 40 min. One blank followed by six replicates of a single calibration standard solution of Ambrisentan was injected to check the system suitability. To ascertain the systems suitability for the proposed method, the parameters such as theoretical plates, peak asymmetry, retention time and parameters were taken. [13]

Specificity

The effect of wide range of excipients and other additives usually present in the formulations ofAmbrisentan in the determinations under optimum conditions was investigated. The specificity of the RP-HPLC method was established by injecting the common excipients such as lactose anhydrous, microcrystalline cellulose and magnesium stearate have been added to the placebo solution and injected and tested. [14]

Linearity

The linearity graphs for the proposed assay methods were obtained over the concentration range of 6-30 µg/mLof Ambrisentan. Method of least square analysis was carried out for getting the slope, intercept and correlation coefficient.A calibration curve was plotted between concentration and area response.

Precision

Intraday and interday precision study of Ambrisentan was carried out by estimating corresponding responses 3 times on the same day and on 3 different days for the concentration of 10µg/mL.

Acceptance: The percent relative standard deviation (% RSD) shouldnot more than 2.0.

Ccuracy (Recovery Studies)

The accuracy of the method was determined by calculating recovery of Ambrisentan by the method of addition. Known amount of Ambrisentan at 25%, 50% and 100% was added to a pre-quantified sample solution. The recovery studies were carried out in the tablet in triplicate each in the presence of placebo. **Acceptance:** The mean percentage recovery of Ambrisentan at each level was not less than 99.84% and not more than 100.2%.

LOD and LOQ

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit of Quantitation were calculated using following formula LOD = 3.3(SD)/S and LOQ= 10 (SD)/S, where SD = standard deviation of response (peak area) and S = slope of the calibration curve. [15]

Robustness

The Robustness was evaluated by the analysis of Ambrisentan under different experimental conditions such as making small changes in flow rate (± 0.2 mL/min), detection wavelength (±5nm) and mobile phase composition (±5%).

RESULTS AND DISCUSSION

Optimization of HPLC Method

An accurate RP-HPLC method was developed in order to determine Ambrisentan in tablet formulation using the optimized chromatographic conditions. The mobile phase consisting of 10 mM phosphate buffer (pH-6.0): acetonitrile (50:50, %v/v at 1.2 $mL.min^{-1}$ flow rate and detection wavelength of 226nm was optimized which gave sharp peak, minimum tailing factor with short runtime for Ambrisentan. The retention time for Ambrisentan to be 2.897minutes. System suitability parameters and optimized chromatographic conditions are shown in Table 1 and the chromatograms of several trails are presented in Fig.1 and 2. In the case of trail 1**,** System suitability parameters were not satisfied and trail 2 system suitability parameters were satisfied. The absorption curve of Ambrisentan and Tadalafil showing detection wavelength is given in Fig.3

Validation of HPLC Method

The specificity was studied for the examination of the presence of interfering components, while the comparison of chromatograms there was no interference from placebo (Figure 4) with sample peak. They do not disturb the elution or quantification of Ambrisentan. Furthermore the well-shaped peaks also indicate the specificity of the method. Therefore, it was concluded that the method is specific. The specificity results are summarized in Table 2.The calibration curve for Ambrisentan was found to be linear over the range of 6-30µg/mL. The data of the calibration is shown in Table. 3 and the chromatograms for calibration are presented in Figure 7 - 11. The regression equation for Ambrisentan internal standard peak area ratio was found to be $Y = 0.0737x + 0.0471$with correlation coefficient, $R^2 = 0.998$ which indicates this method has good linearity. The data for regression analysis of the calibration result is presented in Table 4. The linearity graphs are shown in Figure 12 for Ambrisentan. Precision was studied to find out intra and inter day variations in the test methods of Ambrisentan for six times on the same day and different day. The intra-day and inter-day precision obtained was % RSD (< 2.0) indicates that the proposed method is quite precise and reproducible and results are shown in Table 5 for Intra-day and Table 6 for Inter-day precision studies. The representative chromatograms are depicted in Figure 13 for Intra-day and Figurer 4.16 for Inter-day precision studies. Recovery studies of the drug were carried out for the accuracy parameter at three different concentrations levels i.e. multiple level recovery studies. A known amount standard was added into pre-analysed sample and

subjected them to the proposed HPLC method. The % recovery was found to be within the limits as listed in Table 7. The representative chromatograms for recovery studies at various levels were shown in Figure 15-17 for Recovery levels of 25%, 50% and 100% respectively. Generally the mean percentage recovery of Ambrisentan at each level was not less than 98 % and not more than 102 %. Robustness was done by small changes in the chromatographic conditions like mobile phase flow rate, detection wavelength, mobile phase composition etc. It was observed that there were no marked changes in the chromatograms. In fact the parameters are within the limit which indicates that the method has robustness and suitable for routine use. The Robustness results are presented in Table 8. The limit of detection (LOD) and limit of quantitation (LOQ) was calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approximating the LOD and LOQ. The limit of detection (LOD) of Ambrisentan is1.3267µg/mL and the limit of quantitation (LOQ) of Ambrisentan is4.0206µg/mL which shows that this method is very sensitive. The results are shown in Table 9.The developed method was applied to the assay of CIALIS tablets containing Ambrisentan. The assay results of different injections of the sample were found to be within the proposed limits. The mean assay value was found to be 100.93% for Ambrisentan. The results were very close to labelled value of commercial tablets. [19-17] The representative sample chromatogram of Ambrisentan is shown in Figure 18 respectively. The experimental results are given in Table 10.

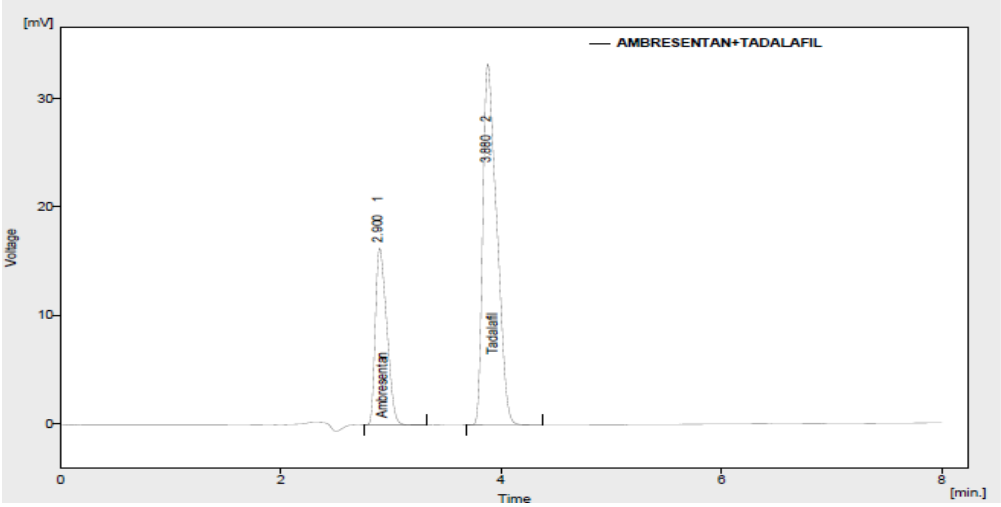

Fig. 1: Chromatogram of Ambrisentan and Tadalafil trial-1

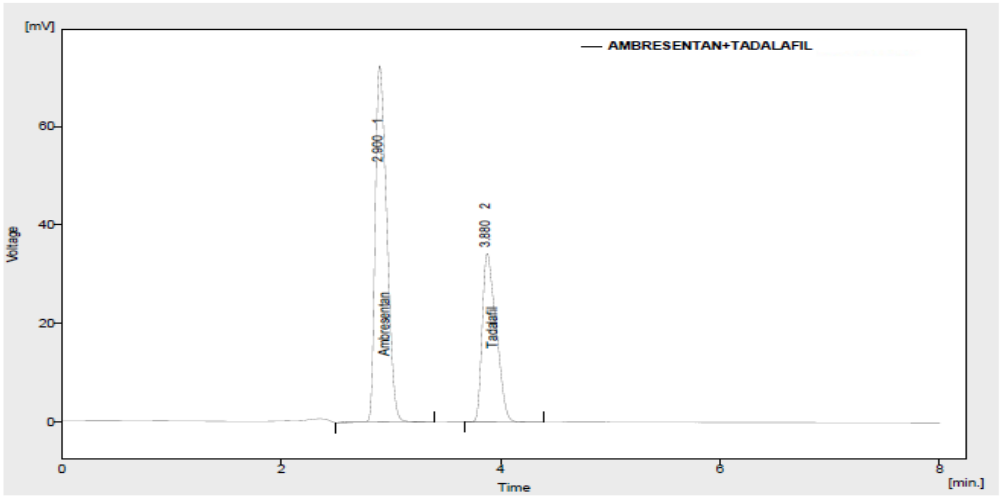

Fig.2: Chromatogram of Ambrisentan and Tadalafil trial-2

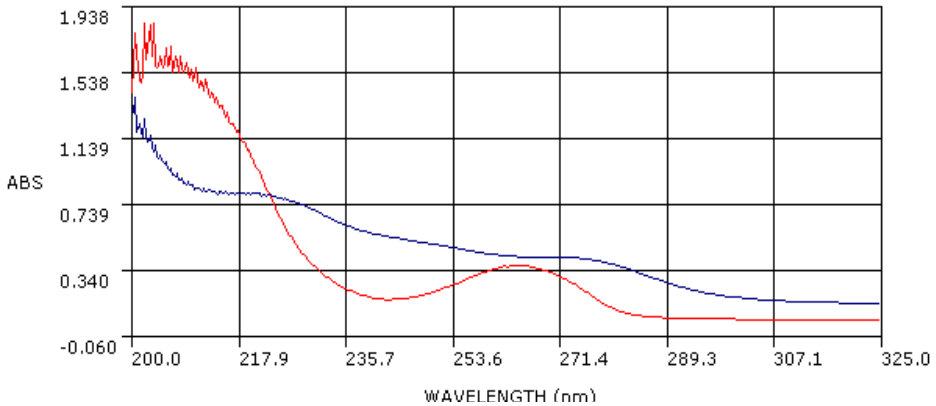

Fig. 3: Absorption Spectra of Ambrisentan and Tdalafil.

Table 1: Optimized Chromatographic Conditions and System Suitability Parameter.

Parameter	**Chromatographic conditions**
Instrument	SHIMADZU LC-20AT Prominence liquid chromatograph
Column	WELCHROM C_{18} Column (4.6 X 250mm, 5µm)
Detector	SHIMADZU SPD-20A Prominence UV-Vis detector
Diluents	10mM Phosphate Buffer(pH 6.0) : Acetonitrile (50:50, v/v)
Mobile phase	10mM Phosphate Buffer(pH 6.0) : Acetonitrile (50:50, v/v)
Flow rate	1.2mL/min.
Detection wave length	UV at 226nm.
Run time	8 minutes
Column back pressure	124 kgf
Temperature	Ambient temperature(25^0C)
Injection Volume	20µL
Retention time (t_R) for AMB,TADA	2.9 and 3.8 respectively
Theoretical plates[th.pl] (Efficiency)$^{\$}$	3236
Resolution*	7.98
Tailing factor (asymmetry)$^{\#}$	1.28

Acceptance criteria (Limits):

$Theoretical Plates >3000, *Resolution >2.0, #Peak Asymmetry <1.5.

Table 2: Specificity study.

Name of the solution	Retention time, (t_R)min.
Mobile phase	No peaks
Placebo	No peaks
Solution containing a concentration of AMB, 6µg/mL.	Peaks at 2.97 min

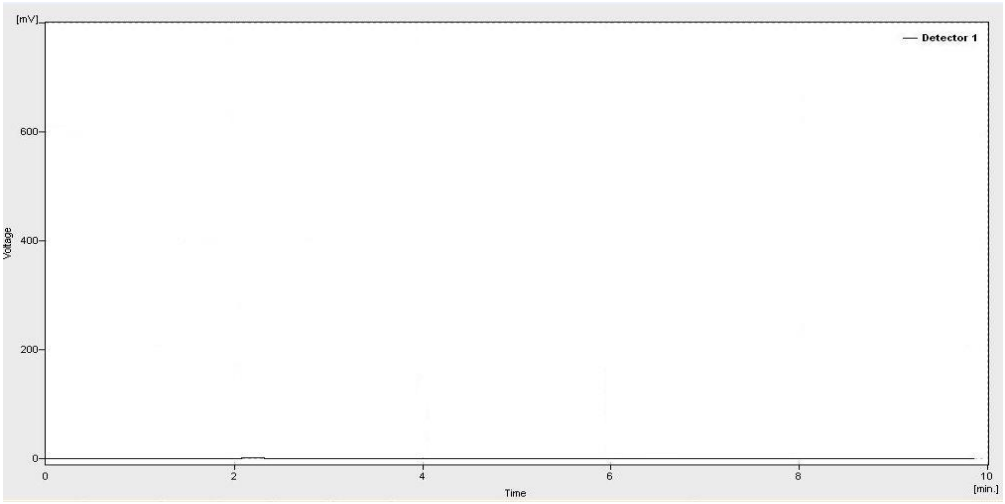

Fig. 4: Chromatogram of placebo.

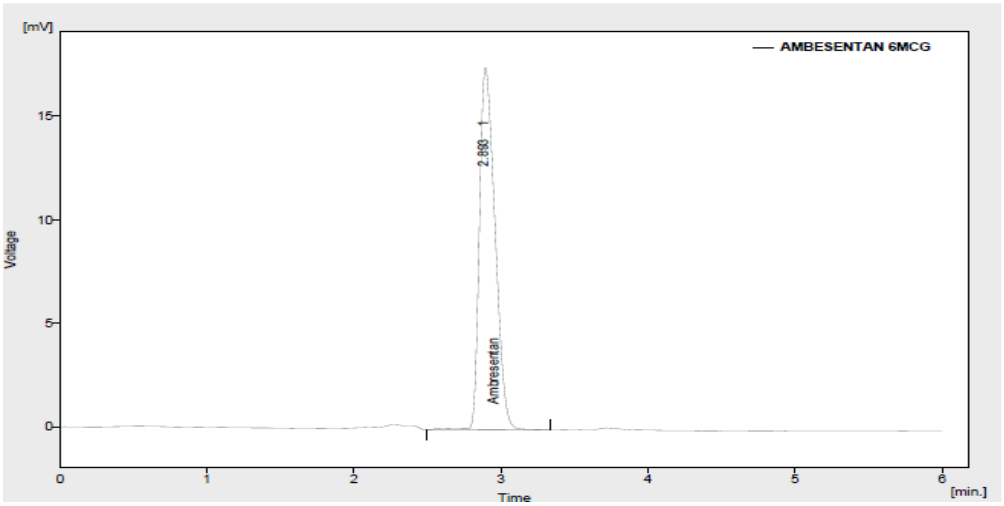

Fig. 5: Chromatogram of Ambrisenta.

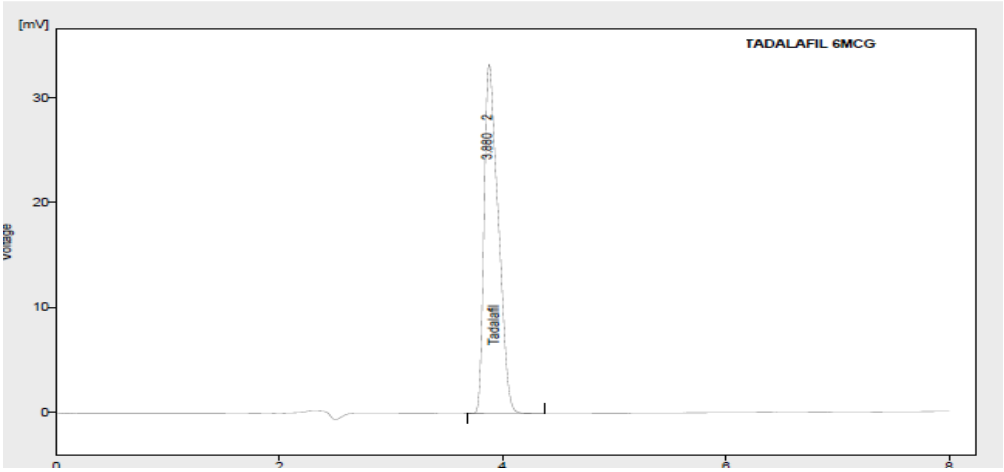

Fig. 6: Chromatogram of Tadalafil.

Table 3: Chromatographic Results.

Drug	Concentration,µg/ml	Retention time, min.		Peak area ratio, mV.s.	Peak Asymmetry[#]	Plate count[$]
		Amb	Tada			
Ambrisentan+ Tadalafil	6+6	2.897	3.883	0.500	1.28	3236
	12+6	2.897	3.883	0.981	1.28	3233
	18+6	2.897	3.883	1.390	1.28	3232
	24+6	2.897	3.883	1.823	1.28	3230
	30+6	2.897	3.883	2.218	1.28	3236

Acceptance criteria (Limits):[#]Peak Asymmetry < 1.5, [$] Plate count > 3000.

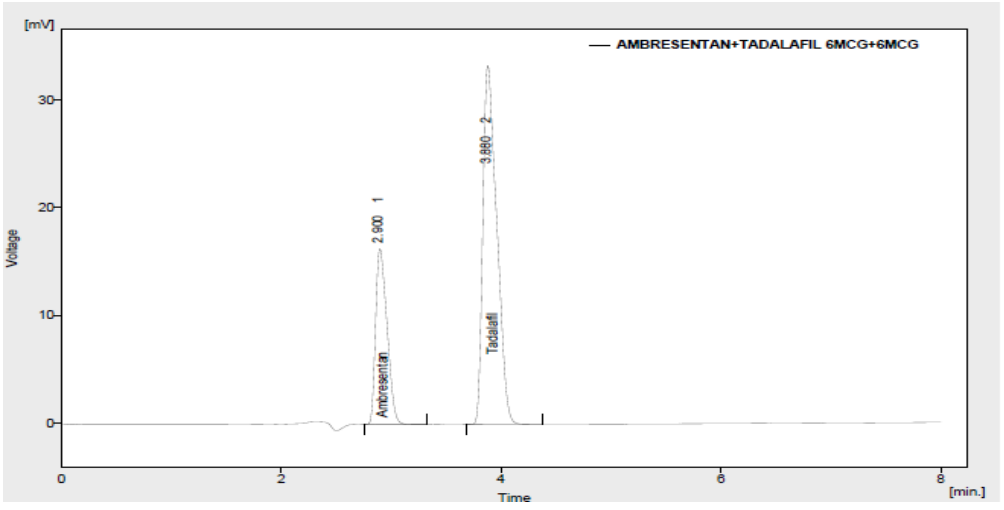

Fig. 7: Chromatogram ofAmbrisentan (6µg/mL) and Tadalafil (6µg/mL)

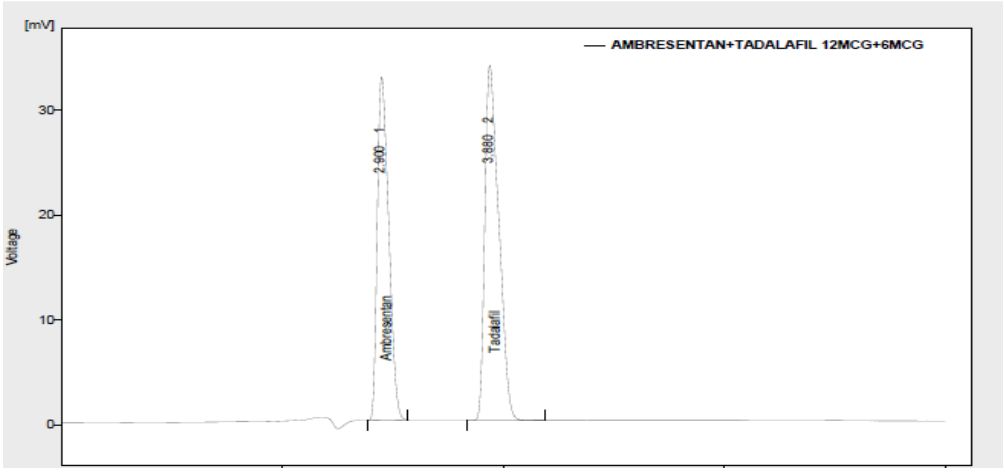

Fig. 8: Chromatogram ofAmbrisentan (12µg/mL) and Tadalafil (6µg/mL).

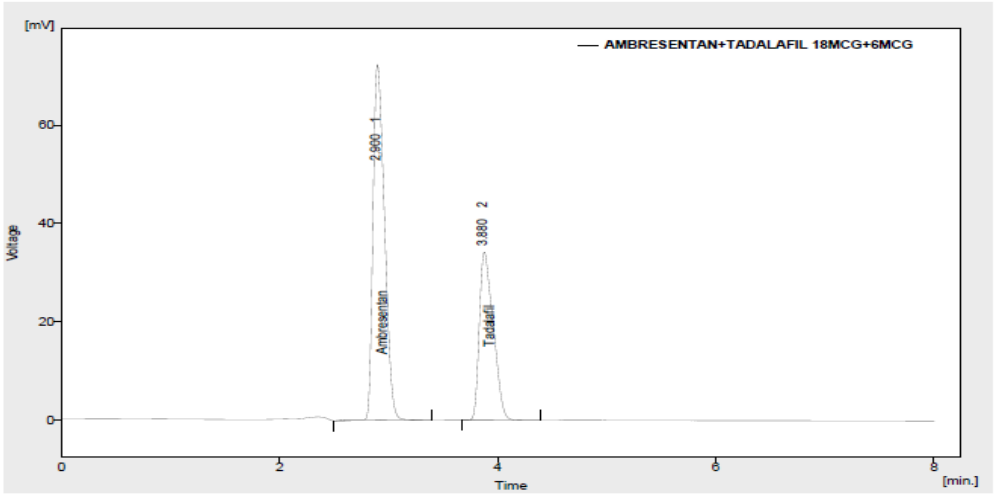

Fig. 9: Chromatogram ofAmbrisentan (18µg/mL) andTadalafil (6µg/mL).

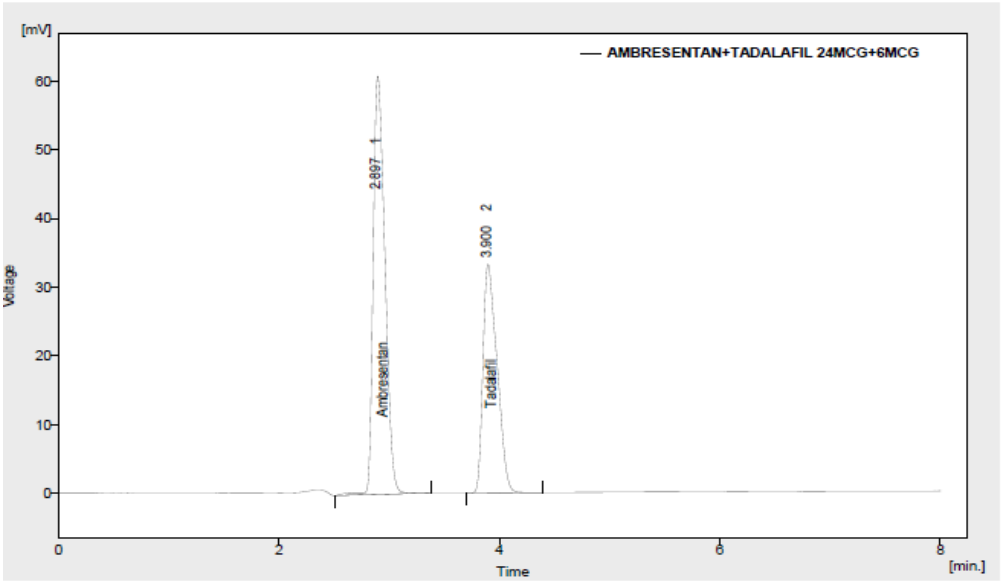

Fig. 10: Chromatogram ofAmbrisentan (24µg/mL) andTadalafil (6µg/mL).

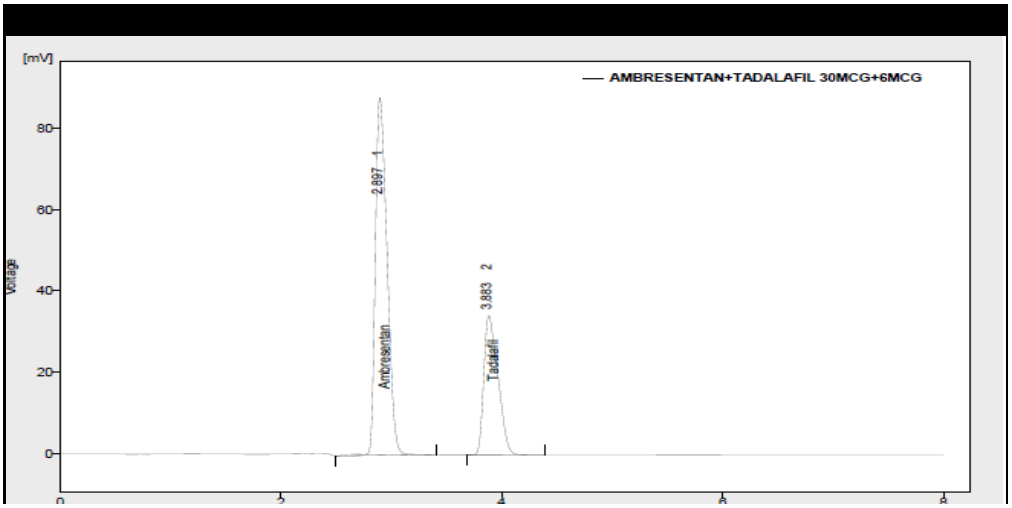

Fig. 11: Chromatogram ofAmbrisentan (30µg/mL) and Tadalafil (6µg/mL).

Table No.4: Linearity Regression results of Ambrisentan

Parameter	**Ambrisentan**
Detection wavelength(λ_{max})	UV at 226 nm
Linearity range (µg/mL)	6-30µg/mL
Regression equation (Y = aX + b)	y = 0.0737x + 0.0471
Slope(a)	0.0737
Intercept(b)	0.0471
Standard error of slope (S_a)	0.00163
Standard error of intercept (S_b)	0.02962
Standard error of estimation (S_y)	0.04092
Regression coefficient (R^2)	0.998
Percentage range of errors (Confidence limits) 0.005 significance level 0.001 significance level	 0.6793 1.0123

[#]Average of 6 determinations; acceptance criteria < 2.0.

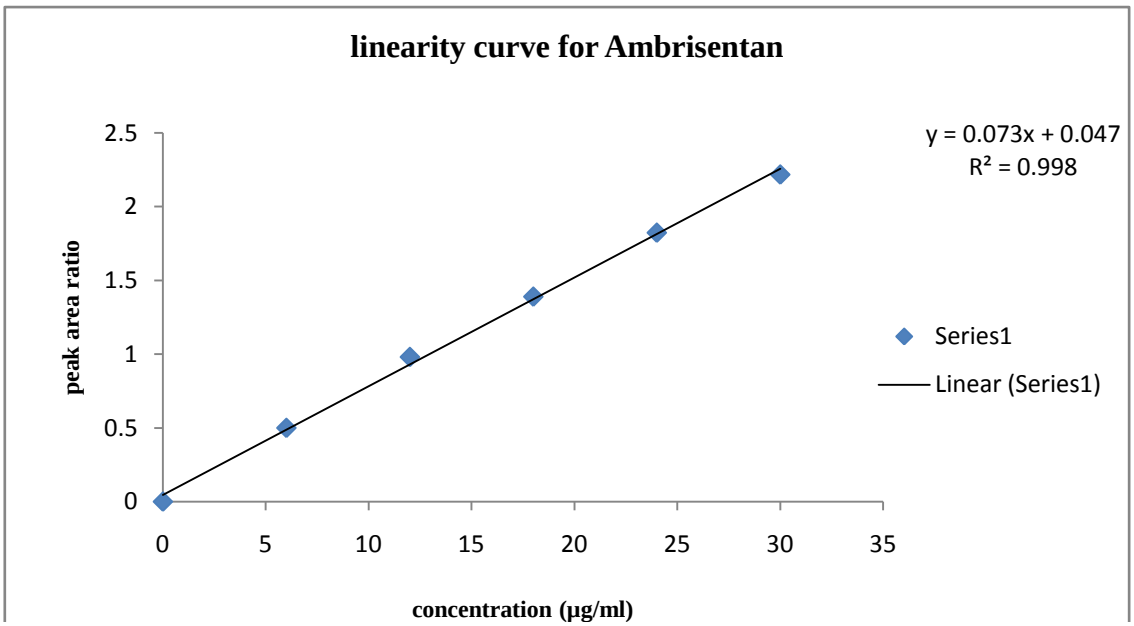

Fig. 12: Linearity curve for Ambrisentan.

Table 5: Results of Intra-Day Precision Study.

Trial No.	Peak area ratio	Concentration (µg/ml)	Amount (mg/ml)	% Assay
1	0.943	12.15	10.08	100.8
2	0.945	12.18	10.11	101.1
3	0.943	12.15	10.08	100.8
4	0.945	12.18	10.11	101.1
5	0.942	12.14	10.08	100.7
6	0.945	12.18	10.11	101.1
Mean	0.943	12.163	10.095	100.93
S.D	0.0013	0.018	0.016	0.186
%RSD	0.140	0.153	0.162	0.184

[#] Acceptance criteria < 2.0

Table 6: Results of Inter-Day Precision Study.

Trail No.	Peak area ratio	Concentration (µg/ml)	Amount (mg/ml)	% Assay
1	0.932	12.0	9.96	99.6
2	0.930	11.97	9.94	99.4
3	0.932	12.0	9.96	99.6
4	0.932	12.0	9.96	99.6
5	0.930	11.97	9.94	99.4
6	0.932	12.0	9.96	99.6
Mean	0.931	11.98	9.95	99.53
S.D	0.0010	0.0164	0.0103	0.1032
%RSD	0.1108	0.1037	0.1037	0.1037

[#] Acceptance criteria < 2.0

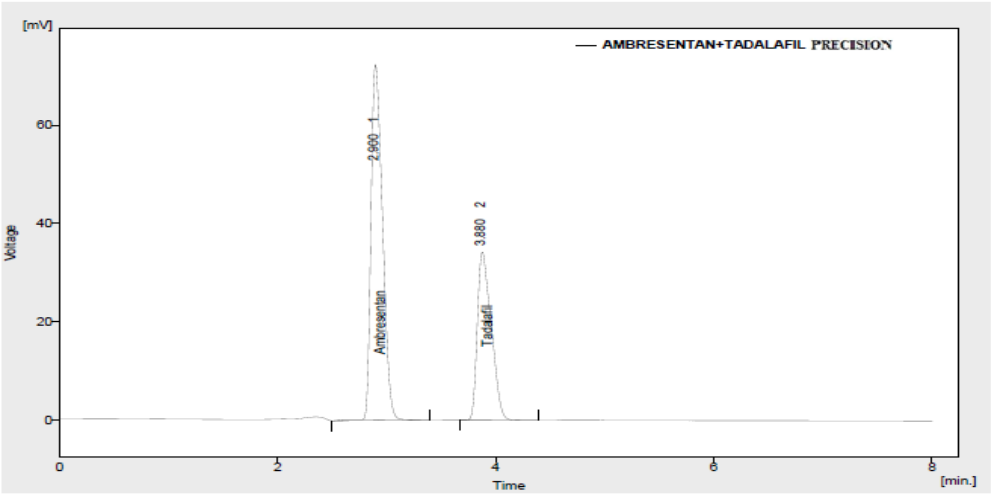

Fig. 13: Representative Standard chromatogram for precision (intra-day).

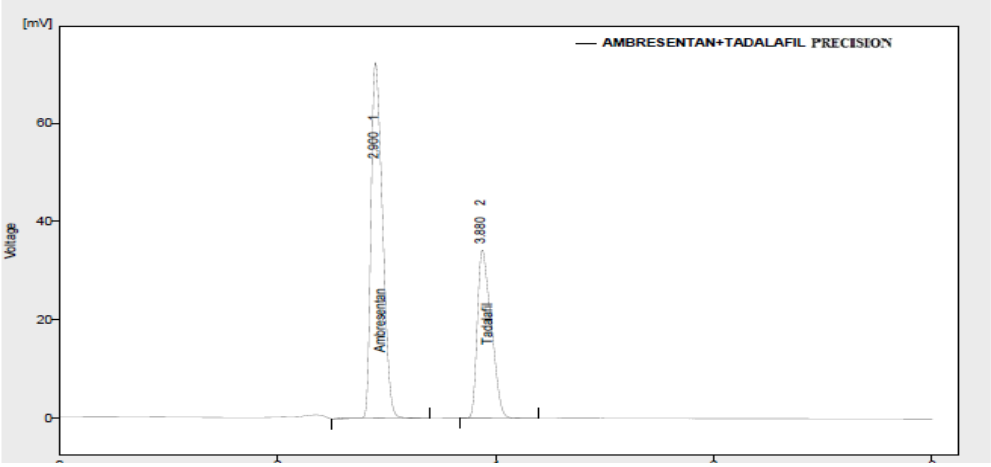

Fig. 14: Representative Standard chromatogram for precision (inter-day).

Table 7: Recovery Studies.

S.No.	Level of spiking of standard	Amount of standard added to pre analyzed samples (mg)	Amount found (mg)	% recovery*
1	25%	2.5mg	12.48	100
2	50%	5.0mg	14.98	99.86
3	100%	10mg	19.98	99.9

[#] Acceptance criteria < 2.0,[*]average of triplicate injections.

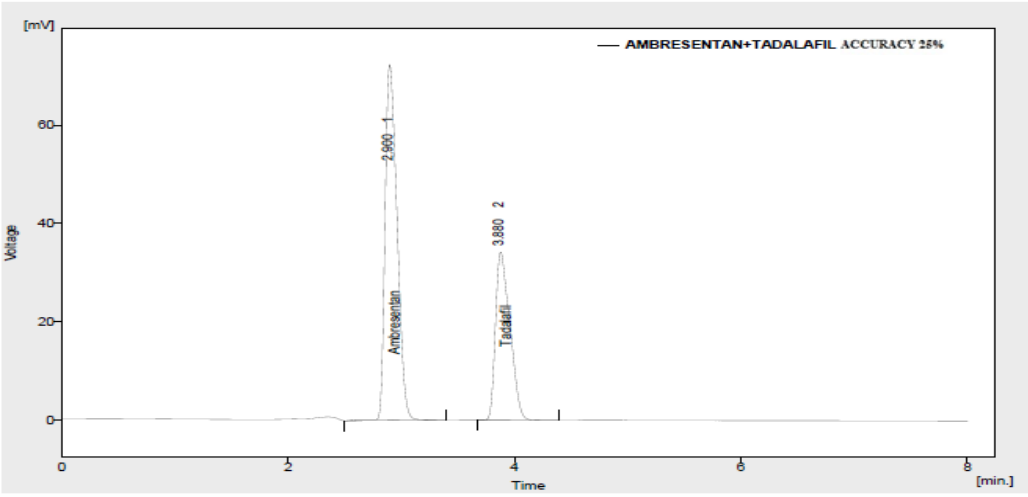

Fig. 15: Chromatogram for Accuracy- 25% level.

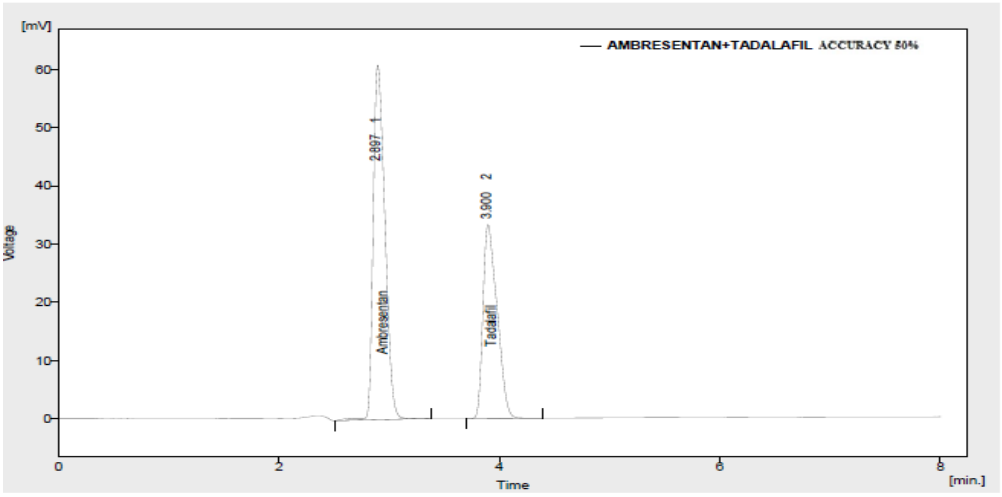

Fig. 16: Chromatogram for Accuracy- 50% level.

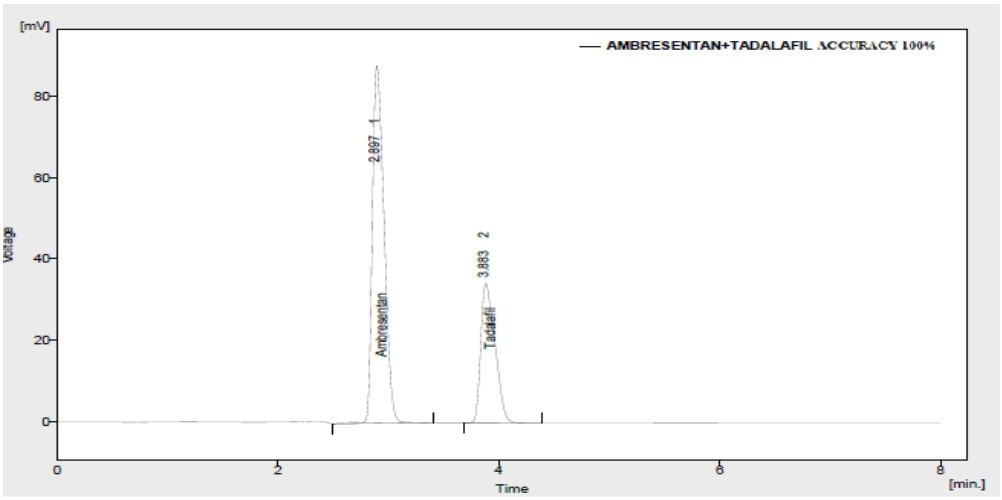

Fig. 17: Chromatogram for Accuracy- 100% level.

Table 8: Results of robustness.

S. No.	Parameter[a]	Used	Retention time (t_R), min.	Plate count[$]	Peak asymmetry[#]	Remarks
1.	Flow rate (±0.2 mL/min)	1.0 mL/min	2.720	3236	1.28	*Robust
		1.4 mL/min	2.852	3233	1.25	*Robust
2.	Detection wavelength (±5 nm)	221 nm	2.897	3236	1.23	Robust
		231 nm	2.962	3225	1.22	Robust
3.	Mobile phase composition (±2 % v/v)	52:48, %v/v	2.568	3216	1.25	*Robust
		48:52, %v/v	2.668	3236	1.26	*Robust

Acceptance criteria (Limits):[#]Peak Asymmetry < 1.5, [$] Plate count > 3000, *significant change in Retention time.

Table 9: LOD and LOQ.

Drug	LOD	LOQ
Ambrisentan	1.3267µg/mL	4.02060 µg/mL

Table 10: Assay Results.

S.NO	Brand name	Label claim	Amount found*	% Assay	% RSD
1.	CIALIS	10mg	10.095	100.93	0.184

*****Average of 6 determinations; %RSD is relative standard deviation.

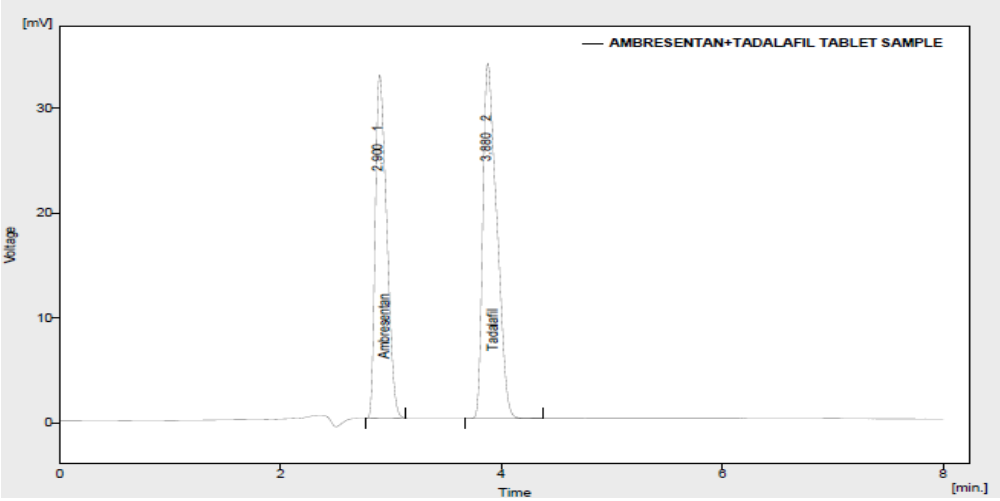

Fig. 18: Chromatogram for assay of Tablet.

CONCLUSION

The present method demonstrated the estimation of Ambrisentan is available as tablet dosage forms using RP-HPLC. The linearity of the proposed method was in the range of 6-30 µg/mL. The Limit of detection and Limit of Quantitation for estimation of Ambrisentan were 1.326µg/mL and 4.020µg/mL. The developed RP-HPLC method for the quantification of Ambrisentanwas found to be simple, specific, highly sensitive, rapid, economical, precise and very accurate with robustness. The developed method has various advantages like good linearity, less retention times and less solvent consumption which makes the method economical. So, the developed method may be successfully applied for the determination of Ambrisentanin pharmaceutical quality control laboratories for routine analysis.

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