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Development and validation of novel stability-indicating RP-HPLC method for estimation of related substances and degradation products in ambrisentan

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ABSTRACT

A simple, specific, precise, linear, accurate, reproducible and stability indicating RP-HPLC method was developed for the quantative determination of related subatances and degradation products of Ambrisentan. The chromatographic separation was achieved by using Waters symmetry C_{18} column. The drug substance was subjected to stress conditions of hydrolysis (acid, base and water), oxidation, photolysis and thermal degradation as per International Conference on Harmonization (ICH) prescribed stress conditions to show the stability-indicating power of the method. Significant degradation was observed during acid, base and water hydrolysis studies. The mass balance of Ambrisentan was close to 100% in all the stress condition. In the developed HPLC method, the resolution between Ambrisentan and its process-related impurities was found to be greater than 5.0. The limit of detection (LOD) was in the range of 13.6 to 31.2 ng/mL and limit of quantification (LOQ) found to be in the range of 58.4 ng/mL to105.6 ng/mL for Ambrisentan and their impurities. The method was validated for accuracy, linearity, precision, specificity, robustness, and detection and quantification limits, in accordance with ICH guidelines.

KEYWORDS

Ambrisentan;
Reverse phase liquid chromatography;
Stability-indicating method.

INTRODUCTION

Ambrisentan [(+)-(2*S*)-2-[(4,6-dimethylpyrimidin-2-yl)oxy]-3-methoxy-3,3-diphenylpropanoic acid] is used for the treatment of pulmonary arterial hypertension (PAH) to improve exercise ability and delay clinical worsening[1-3]. Letairis is the brand name for Ambrisentan, an endothelin receptor antagonist that is selective for the endothelin type-A (ET_A) receptor[4-8, 3]. Letairis is available as 5 mg and 10 mg film-coated tablets for once daily oral administration. It has a molecular formula of $C_{22}H_{22}N_2O_4$ and a molecular weight of 378.42. The molecular structure of Ambrisentan contains a single chiral center with two possible enantiomeric forms (R-,S-enantiomers). The active substance is the resolved S-enantiomer that in non-clinical studies has been shown to be the most active and has the following structural formula[3]. Ambrisentan is a white to

off-white, crystalline solid. It is a carboxylic acid with a pKa of 4.0. Ambrisentan is practically insoluble in water and in aqueous solutions at low pH. Solubility increases in aqueous solutions at higher pH. In the solid state Ambrisentan is very stable, is not hygroscopic, and is not light sensitive[3]. The literature survey reveals that, Few analytical methods were reported for the quantification of Ambrisentan in plasma and application to rat pharmacokinetic study. The reported analytical method employs the quantification by LC-ESI-MS/MS and UV-spectrophotemetry[9,10]. Rapid determination of Ambrisentan enantiomers by enantioselective liquid chromatography using cellulose-based chiral stationary phase in reverse phase mode is available[11]. No HPLC methods were reported in major pharmacopeia like USP, EP, JP and BP. Therefore, it is felt to develop stability indicating HPLC method for determination of related substances and for quantitative estimation of Ambrisentan in bulk drugs. Hence, an attempt has been made to develop an accurate, specific and reproducible method for the determination of Ambrisentan and all the six impurities in bulk drug samples and along with method validation as per ICH guidelines[12-16].

EXPERIMENTAL

Chemicals and reagents

Ambrisentan and its impurities (Figure1) were provided by MSN Laboratories Pvt Limited, R&D Center, India. Acetonitrile, Potassiumdihydrogenphosphate, Orthophosphoric acid, Hydrochloric acid, Sodium hydroxide and Hydrogen peroxide were purchased from Rankem (Mumbai, India) and used for the studies. All chemicals and reagent used were of HPLC and AR grades, Millipore Milli Q plus (Bangalore, India) purification system was used to prepare high pure water.

Instrumentation

The method development attempts were performed on Agilent 1200 series LC system equipped with a Diode Array Detector (DAD) and data was collected, processed using Ez chrom Elite software. Forced degradation studies and the method validation was performed by using Waters e2695 separation module LC system with 2998 Photodiode Array Detector (PDA) and data were collected, processed using Empower software.

Chromatographic conditions

The chromatographic separation was optimized using Waters symmetry C-18 column with the dimension of 250 mm x 4.6 mm and 5 µm as particle size. A gradient elution was involved with the 2.72 gr Potassium di hydrogen ortho phosphate in 1000 mL of Milli-Q-water (100% v/v) and adjusted its pH 3.0 with dilute ortho phosphoric acid as a mobile phase-A and acetonitrile: water in the ratio of 90:10 (*v/v)* as mobile phase B. The HPLC gradient program was set as: time/% mobile phase B: 0/55, 10/55, 25/65, 33/65, 46/75, 52/75, 53/55 and 60/55. The flow rate of the mobile phase and the column temperature was set as 1.0 mL min^{-1} and 25°C respectively. The detection wave length was optimized at 210 nm. The column loading was finalized as 8 µg of Ambrisentan in 10 µL injection volume. A mixture of Mobile phase-A and Acetonitrile in the same ratio was used as diluent.

Sample preparation

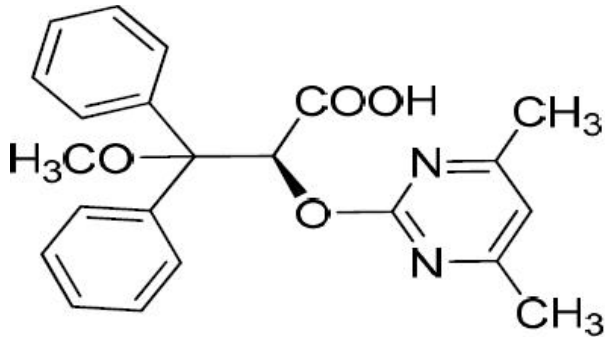
Ambrisentan solution was prepared at target analyte concentration (TAC) which is 800 µg mL^{-1} in the diluent for related substances. A stock solution with the blend of Sulphonyl pyrimidine impurity, Hydroxy acid impurity, Hydroxy ester impurity, Benzophenone impurity, Pyrimidine ester impurity and Vinyloxy impurity were also prepared in diluent for the preparation of system suitability solution with around 0.15 % *w/w* of each impurity at TAC of Ambrisentan.

RESULTS AND DISCUSSION

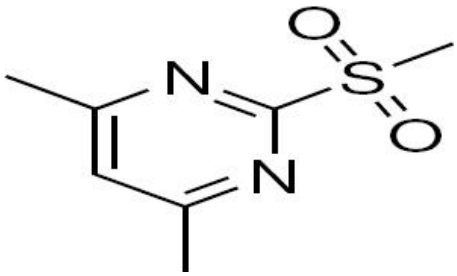
Method development and optimization

The HPLC method carried out in this study aimed to develop a suitable chromatographic system, which is capable of eluting and resolving Ambrisentan from its process related impurities and degradation products that comply with the general requirements for system suitability with good baseline. An following method development conditions are slected based on physical and chemical properties (pKa value, solubility, etc) of Ambrisentan[1-3]. Initial attempts for the method development were made in Inertsil ODS-4V (250 mm × 4.6 mm i.d., particle size 5 µm) with Mobile phase-A as

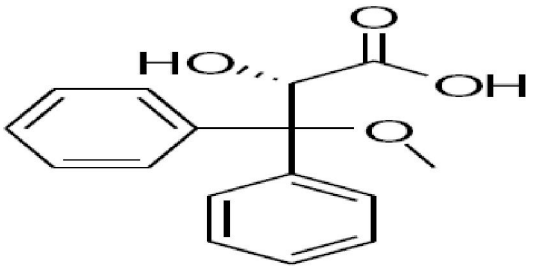
0.01M of potassium dihydrogen ortho phosphate, adjusted at pH to 3.0 adjusted with dilute phosphoric acid and Mobile phase-B as Acetonitrile: water in the ratio of 9:1 (v/v). The gradient as (time (min)/% solution B): 0/55, 10/55, 25/65, 35/65 45/90, 48/90, 49/55, 55/55 at flow rate 1.0 mL·min^{-1}. Column temperature 25ºC, injection volume 10 µL, sample concentration 1 mg. min^{-1}, sample solvent selected as Acetonitrile: buffer in the ratio of 50:50 v/v. With these conditions the separation of the impurities is good, but the baseline was observed to be very poor at 210 nm in the gradient elution. After that we tried with different gradient conditions and finally good base line was observed at selected programme with Intertsil ODS-4 (250 mm × 4.6 mm i.d., particle size 5 µm) column, but in different batches analysis study it is observed that one of the unknown impurity merges with pyrimidine ester impurity. To separate the unknown impurity and pyrimidine ester impurity different stationary phases, various buffers, different pH and different selectivity were used.



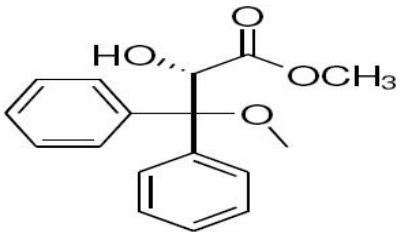
Ambrisentan



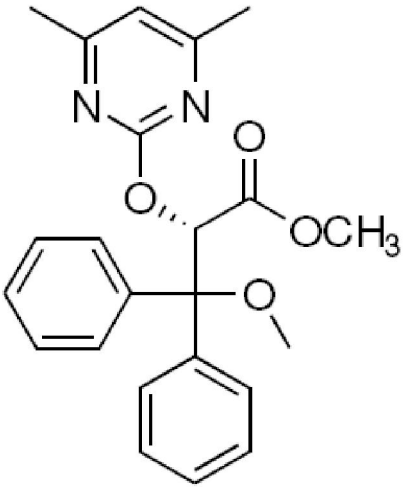
Sulfonyl Pyrimidine impurity



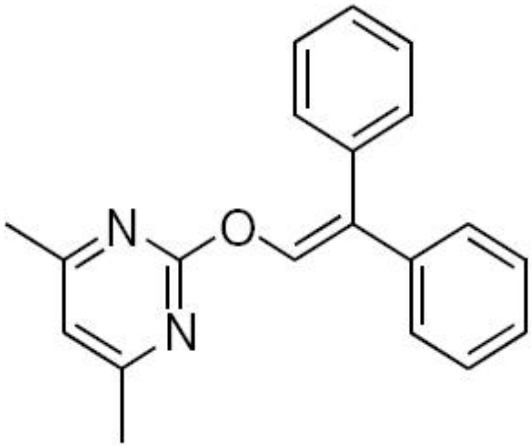
Hydroxy acid impurity



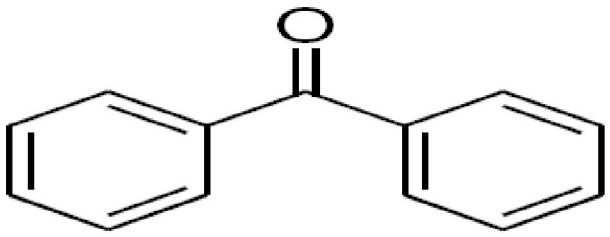
Hydroxy ester impurity



Pyrimidine ester impurity



Vinyloxy impurity



Benzophenone impurity

Figure 1 : Chemical structure of Ambrisentan and its impurities

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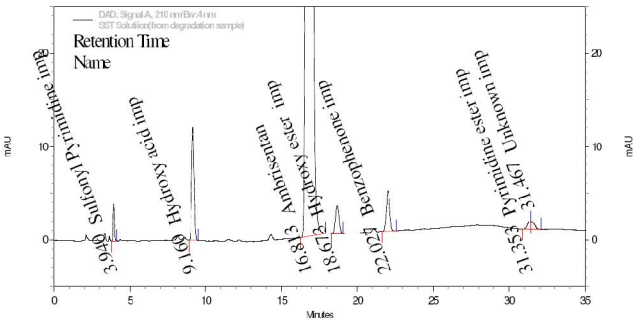
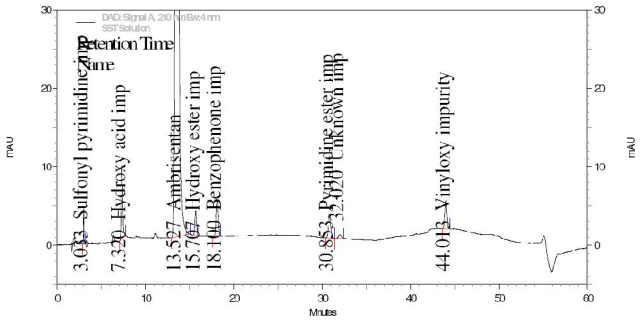
Finally more than two resolution observed between unknown impurity and pyrimidine ester impurity with Symmetry-C_{18} column with the dimension of 250 mm x 4.6 mm and 5 µm as particle size column and also good peak shape with less peak width and the resolution of all the related impurities were satisfactorily (Figure 2). The Column and Acetonitrile were played a key role in the retention times and resolution between impurities. The selected stationary phase is very stable at selected acidic pH 3.0. In the optimized conditions it was observed that the Ambrisentan, Sulphonyl pyrimidine impurity, Hydroxy acid impurity, Hydroxy ester impurity, Benzophenone impurity, Pyrimidine ester impurity and Vinyloxy impurity were well separated with a resolution of greater than 2.

Selectivity/Forced degradation studies

The specificity of the developed LC method for



TYPICAL ZOOMED CHROMATOGRPHIC PATTERN IN INERTSIL ODS-4 250*4.6 MM 5µM.



TYPICAL FULL SCALE CHROMATOGRPHIC PATTERN IN SYMMETRY C_{18} 250*4.6 mm 5µm.

Figure 2 : HPLC column selection chromatograms.

TABLE 1 : Summary of forced degradation results.

Stress conditions		Total impurity degradation	Mass balance	Purity 1 angle	Purity 1 threshold	Purity flag
Normal		0.18%	99.74%	0.120	0.306	No
Acid hydrolysis (After 6 hrs)		7.50%	92.3%	0.120	0.311	No
Base Hydrolysis (After 26 hrs)		6.74%	93.41%	0.143	0.342	No
Oxidation (After 48 hrs)		0.72%	99.34%	0.121	0.316	No
Water Hydrolysis (After 12 hrs)		9.11%	91.56%	0.135	0.319	No
Photo Degradation	UV Direct	0.22%	99.55%	0.089	0.289	No
	UV Indirect	0.20%	99.60%	0.118	0.310	No
	Lux direct	0.19%	99.60%	0.099	0.292	No
	Lux Indirect	0.18%	99.63%	0.116	0.304	No
Thermal at 60°C (10 days)		0.21%	99.65%	0.116	0.310	No
At 75% Relative Humidity (10 days)		0.22%	99.55%	0.098	0.339	No
Under Sunlight (50 hours)		0.38%	99.45%	0.108	0.303	No

Ambrisentan was established in presence of its known impurities (Sulphonyl pyrimidine impurity, Hydroxy acid impurity, Hydroxy ester impurity, Benzophenone impurity, Pyrimidine ester impurity) as process related impurities and Vinyloxy impurity as its degradation product. Forced degradation studies were performed on Ambrisentan to study the stability-indicating property and specificity of the developed method. The relative humidity study stress at 75% relative humidity for 10 days. Under sunlight, stress study was conducted for 50 hours. The photolytic stress studies were performed for UV direct (200 watt hours/square meter), UV in direct (200 watt hours/square meter), Lux direct (1.2 million LUX hours) and Lux in direct (1.2 million LUX hours) as per ICH Q1B[17]. The thermal stress was done at 60 °C for 10 days. The acid stress was performed with 3 N HCl using concentrated sample solution at ambient temperature for 6 hours and base stress was performed with 5 N NaOH for 26 hours at ambient temperature. Further dilution was done to analyte concentration for the quantification of Ambrisentan and its degradants. Water hydrolysis was performed for 12 hours at 60 °C. The oxidation stress was done using 10% hydrogen peroxide for 48 hours at ambient temperature[18]. Degradation was not observed in stressed conditions when the analyte degraded to photolytic, thermal, sunlight, 75% relative humidity and oxidation. The degradation of drug substance was observed only under acid, base and water hydrolysis (Figure 4). All stressed samples were quantified for Ambrisentan and the impurities. Peak purity of stressed of Ambrisentan and the spiked solutions of all the impurities (Sulphonyl pyrimidine impurity, Hydroxy acid impurity, Hydroxy ester impurity, Benzophenone impurity, Pyrimidine ester impurity and Vinyloxy impurity) were checked by using PDA detector. The purity angle was within the purity threshold limit for all the stressed samples, demonstrating the homogeneity of the analyte peak. The impurities content was calculated for all the stress samples against a qualified reference standard. The mass balance (% assay + % of impurities + % of degradation products) was also calculated for all the samples. The summary of the forced degradation was captured in TABLE 1.

System suitability and precision

The system suitability was checked by spiking the all impurities to test sample. The system is deemed to be suitable as the tailing factor < 2.0, Theoretical Plates for Ambrisentan peak is > 3000 and the resolution between closely eluting Ambrisentan and Hydroxy ester impurity impurity>2.0 (TABLE 2) (Figure 3).

Precision is the closeness of agreement between a series of measurements obtained from multiple sampling of same sample under the prescribed conditions. Six individual preparation of Ambrisentan solution was made with each of the known impurities at 0.15 % *w/w* level to TAC. Quantification of individual impurities and Ambrisentan was done for each preparation, the % RSD content and the assay of all the impurities were determined. Method precision was done by repeated experiments with different columns and different instruments in the same laboratory to evaluate the intermediate precision.

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

The LOD and LOQ of an individual analytical pro-

TABLE 2 : System Suitability results

Name	Retention time	USP Tailing	USP Resolution	USP Plate count	Purity 1 angle	Purity 1 threshold
Sulphonyl pyrimidine I Impurity	3.083	1.17	NA	10669	0.304	0.586
Hydroxy acid Impurity	6.937	1.45	19.34	10856	0.549	0.773
Ambrisentan	12.603	1.11	17.18	17789	3.024	3.159
Hydroxy ester Impurity	14.667	1.04	5.46	25541	0.598	0.834
Benzophenone Impurity	16.793	1.04	5.77	34858	0.803	0.821
Pyrimidine ester Impurity	28.716	1.02	28.38	60446	0.704	0.911
Vinyloxy impurity	43.258	1.00	26.87	85112	0.473	0.632

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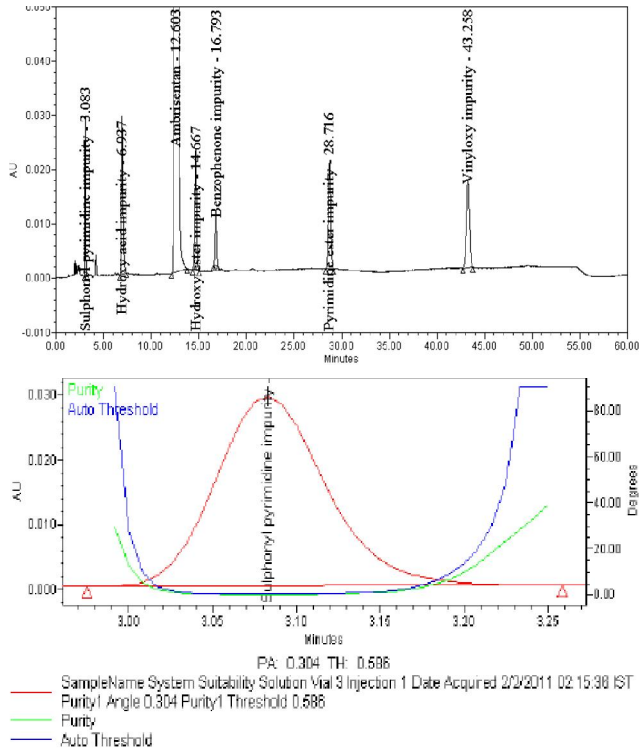
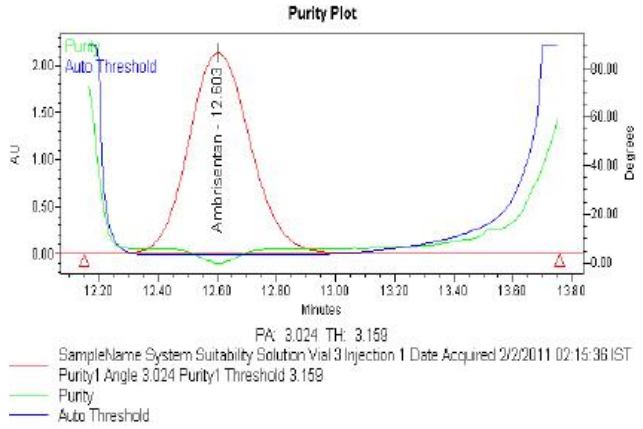
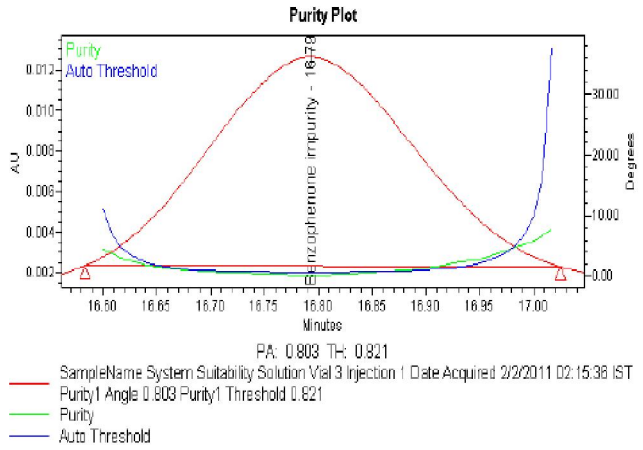


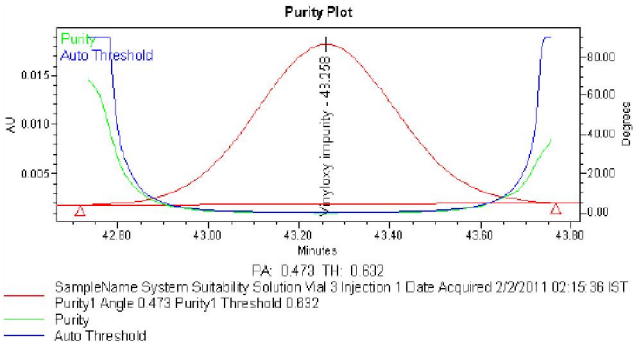
Sulfonyl Pyrimidine impurity peak purity



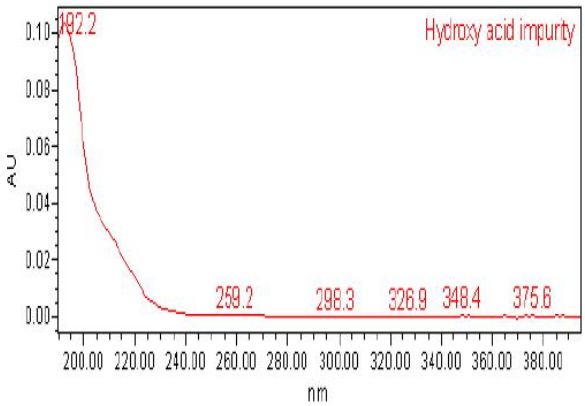
Ambrisentan peak purity



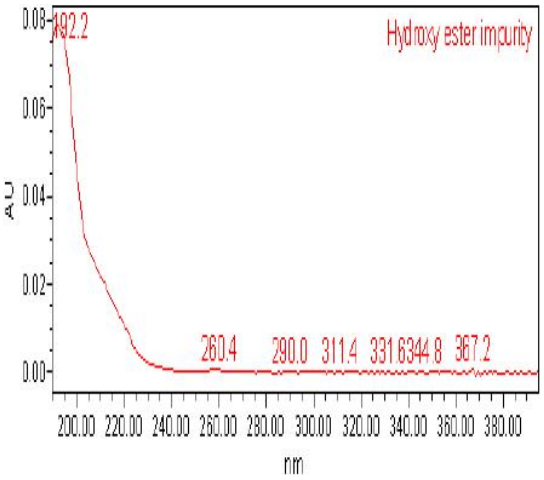
Benzophenone impurity peak purity



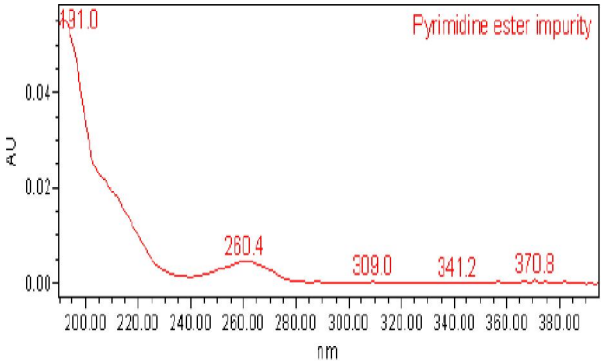
Vinyloxy impurity peak purity



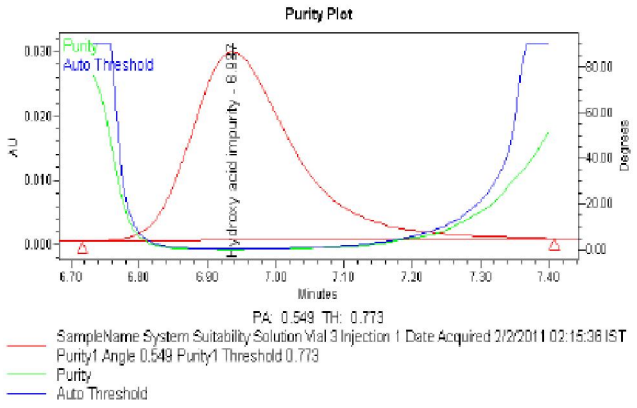
Hydroxy Acid impurity UV- Spectrum



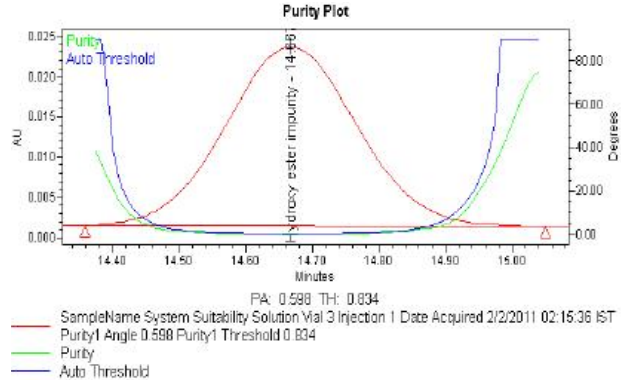
Hydroxy Ester impurity UV- Spectrum



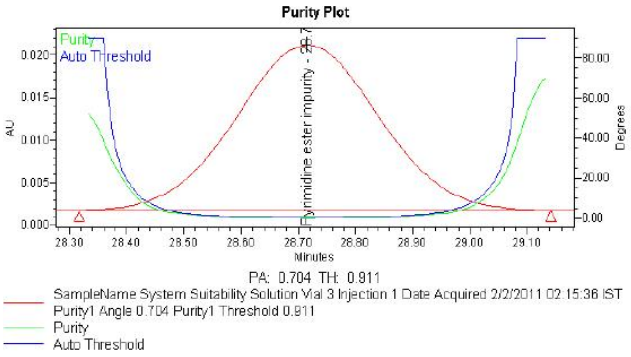
Pyrimidine Ester impurity UV- Spectrum



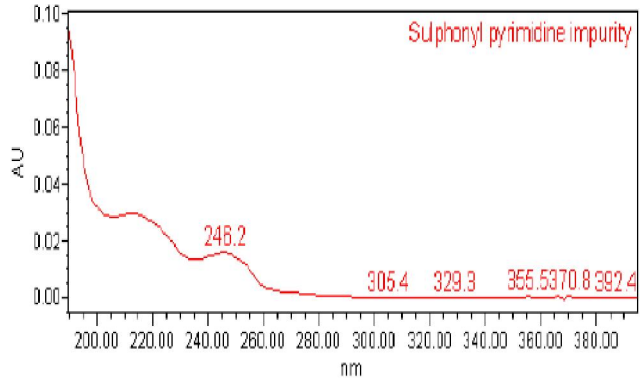
Hydroxy Acid impurity peak purity



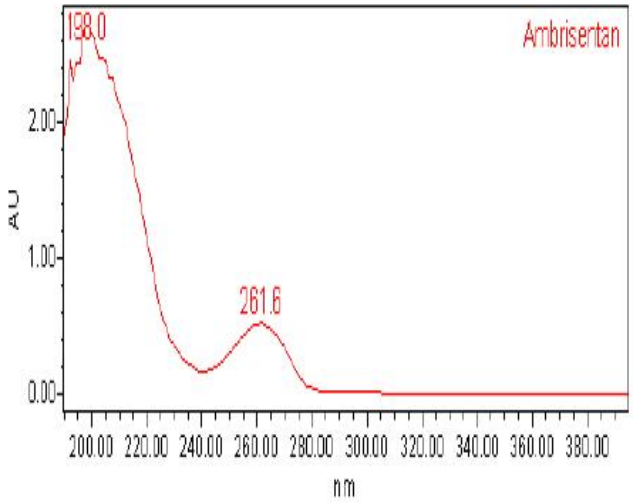
Hydroxy Ester impurity peak purity



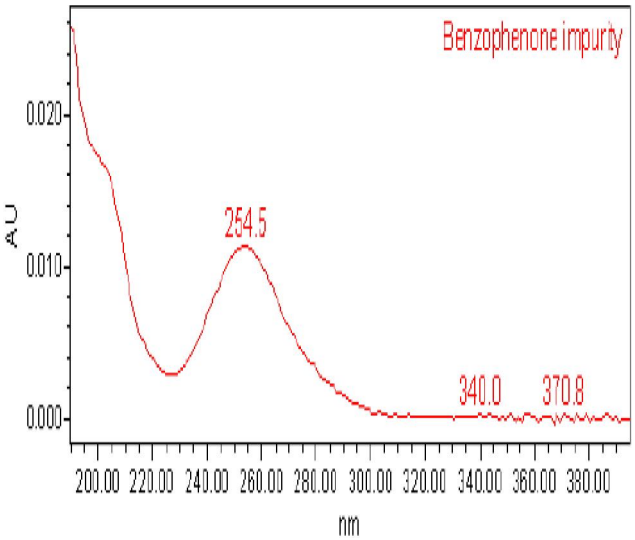
Pyrimidine Ester impurity peak purity



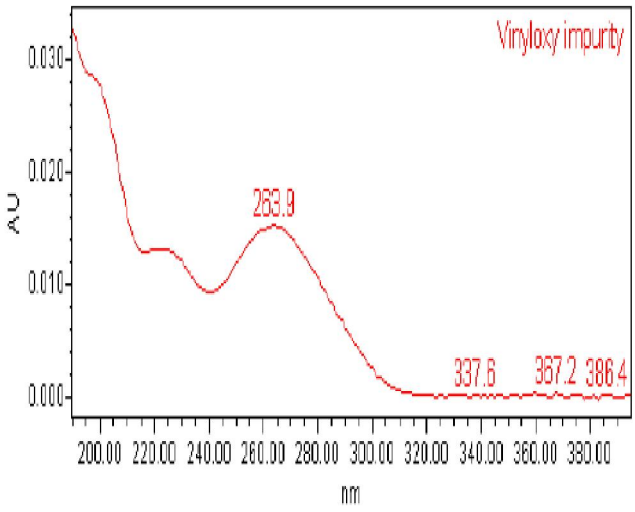
Sulphonyl Pyrimidine impurity UV- Spectrum



Ambrisentan UV- Spectrum



Benzophenone impurity UV- Spectrum



Vi nyloxy impurity UV- Spectrum

Figure 3 : Purity plots and UV spectrums of Ambrisentan and its impurities.

cedure are the lowest amount of analyte in a sample which can be detected and can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ for each of the components were established by attaining signal-to-noise ratio of around 3:1 and 10:1 respectively, from a series of dilute solutions with known concentration. Six individual solutions of known impurities and Ambrisentan were also prepared at LOQ level and the area of impurities and Ambrisentan was recorded. The LOQ precision was established by calculating the % RSD of each known impurity and Ambrisentan peak area. Accuracy of impurities at this level was established by spiking the known LOQ quantities of impurities to the test sample and calculating the recovery of impurities (TABLE 3).

Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the amount of analyte in the sample. To study the linearity at impurity level, the solution of Ambrisentan and its known impurities were prepared in different concentrations from LOQ to 150 % *w/w* of analyte concentration. The slope, intercept and correlation coefficient were calculated (TABLE 4).

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the values obtained

TABLE 3 : Limit Of Detection (LOD) and Limit Of Quantification (LOQ) Results

Compound	Limit Of Detection		Limit Of Quantitation			
	% with respect to analyte concentr-ation	In ng mL^{-1}	% with respect to analyte concentr-ation	In ng mL^{-1}	% of RSD	% of Recovery
Ambrisentan	0.0024	19.2	0.0096	76.8	7.86	Not applicable
Sulfonyl pyrimidine Impurity	0.0022	17.6	0.0088	70.4	3.97	100.7
Hydroxy acid Impurity	0.0035	28	0.0088	70.4	6.10	101.0
Hydroxy ester Impurity	0.0028	22.4	0.0110	88	7.33	98.4
Benzophenone Impurity	0.0017	13.6	0.0073	58.4	8.77	95.0
Pyrimidine ester Impurity	0.0033	26.4	0.0132	105.6	6.44	98.7
Vinyloxy Impurity	0.0039	31.2	0.0131	104.8	4.98	95.4

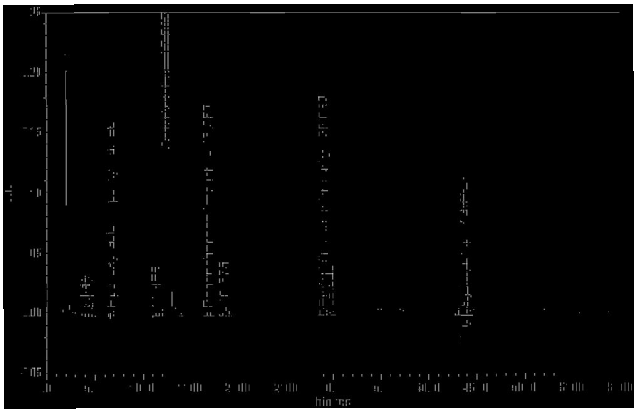
TABLE 4 : Linearity Results

Desired concentration	Sulphonyl Pyrimidine Impurity	Hydroxy Acid Impurity	Hydroxy Ester Impurity	Benzophenone Impurity	Pyrimidine Ester Impurity	Vinyloxy Impurity
LOQ	1066	23219	3253	9901	12568	5181
25	5648	31661	11496	23728	21766	16835
50	8457	42672	20672	34838	32238	29276
75	14370	54710	32480	51375	45415	42752
100	17611	64373	42475	66790	55151	56734
125	22460	75105	53537	80507	67578	65477
150	26578	85387	63730	96275	78307	78148
Slope	175	432	424	590	462	509
Y-Intercept	491	21104	305	7240	9469	3269
Correlation Coefficient	0.9979	0.9997	0.9998	0.9994	0.9994	0.9976

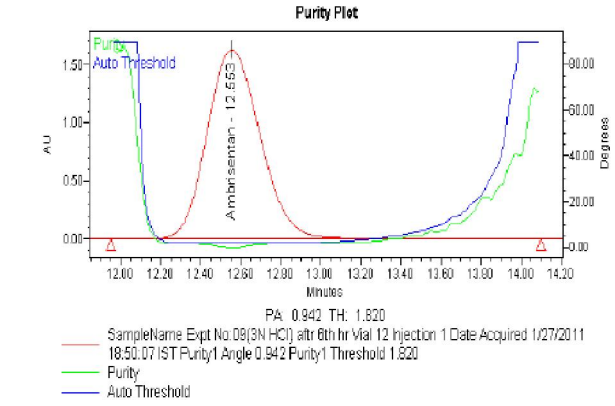
from the method and conventional true value or an accepted reference value. Accuracy of impurities at each

TABLE 5 : Accuracy Results

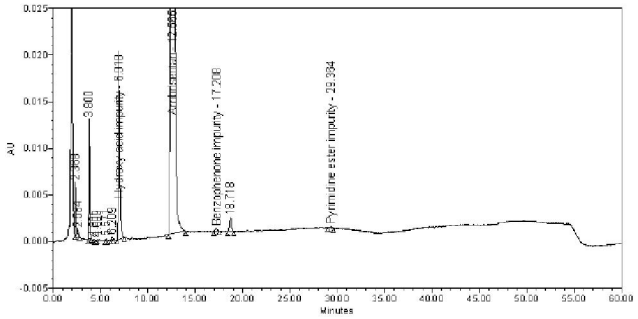
Concentration in %	Area of Sulphonyl pyrimidine Impurity	Area of Hydroxy acid Impurity	Area of Hydroxy ester Impurity	Area of Benzophenone Impurity	Area of Pyrimidine ester Impurity	Area of Vinyloxy Impurity
50	96.2	103.3	98.0	103.3	101.6	105.5
75	109	107.0	102.6	106.6	105.5	104.8
100	100.2	103.2	100.6	106.4	100.1	105.4
125	102.2	103.0	101.5	103.9	101.5	97.7
150	100.8	102.1	100.7	104.6	99.9	97.6
Avg % Recovery	101.7	103.7	100.7	105.0	101.7	102.2



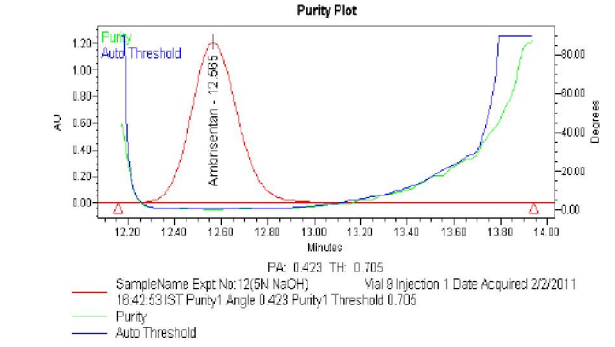
ACID DEGRADATION SAMPLE TYPICAL CHROMATOGRAM:



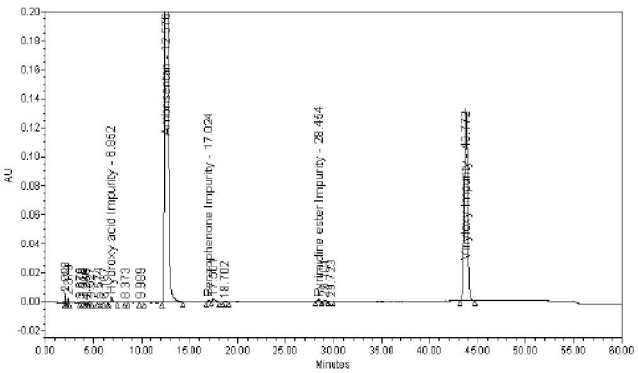
ACID DEGRADATION SAMPLE PEAK PURITY



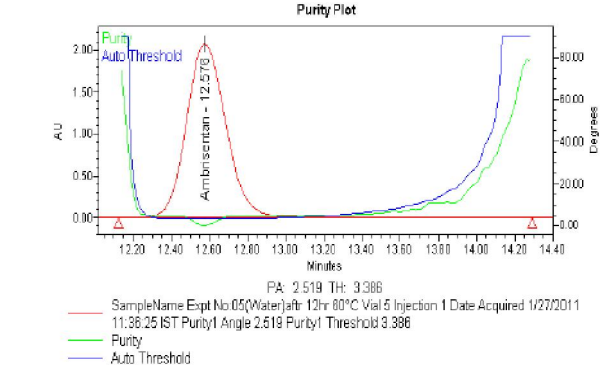
BASE DEGRADATION SAMPLE TYPICAL CHROMATOGRAM



BASE DEGRADATION SAMPLE PEAK PURITY



WATER DEGRADATION SAMPLE TYPICAL CHROMATOGRAM



WATER DEGRADATION SAMPLE PEAK PURITY

Figure 4 : Degradation chromatograms of acid, base and water with peak purity plots.

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level was established by standard addition of the known quantities of impurities to the test sample by calculating the recovery. The study was carried out with five different concentrations at 50 to 150% of the specification level. The recovery of impurities was done by calculating the amount of the impurities spiked to the sample solution (TABLE 5).

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by deliberate variations in method parameters. Small variations were made from original chromatographic conditions to record the resolution between Ambrisentan and its impurities to determine the robustness of the developed method. Six different conditions were studied by a combination of three different small variations. which are flow rate, pH and the column temperature from optimized condition. To determine the robustness of the developed method, experimental conditions were deliberately changed and the resolution (*Rs*) between Ambrisentan and Hydroxy ester impurity were evaluated. The flow rate of the mobile phase was 1.0 mL·min–1. To study the effect of flow rate on the developed method, 0.1 units of flow was changed (*i.e.* 0.9 and 1.1 mL·min–1). The effect of column temperature on the developed method was studied at 20°C and 30°C instead of 25°C. The effect of pH on resolution of impurities was studied by varying ±0.2 pH units (*i.e.* buffer pH altered from 3.0 to 2.8 and 3.2). In the all above varied conditions, the components parameters were held constant.

Solution stability and mobile phase stability

Solution stability and mobile phase stability provides an indication of its reliability between the normal usage and the storage of the solutions used in the method. The solution stability of Ambrisentan was established for 48 hours at room temperature. The solution stability studied by using Ambrisentan sample and injected for every 12 hours. The content of all impurities and Ambrisentan were quantified at each interval up to the study period. The mobile phase stability was also established by quantifying the freshly prepared sample solutions against freshly prepared reference standard solutions for every 12 hours. During the study period the prepared mobile phase was sustained. The recovery of the assay of Ambrisentan and the content of each impurity was calculated against the initial value of the study period.

CONCLUSIONS

The developed stability-indicating analytical method for related substance determination of Ambrisentan and its impurities is precise, accurate, linear and specific. The validation carried out for the method in accordance with the ICH requirements are satisfactory. The developed method can be used conveniently for the routine analysis of production samples and also to check the stability of bulk samples of Ambrisentan during its storage. The same method can also be applied to the drug products to know the information about the impurities and degradation products at lower level.

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