

Design of Experiment Assisted UV-Visible Spectrophotometric and RP-HPLC Method Development for Ambrisentan Estimation in Bulk and Formulations

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Abstract Design of experiment (DOE) approach reinforces the robustness of the method being developed. This was employed for UV-visible (200-400 nm and 400-800 nm respectively) and RP-HPLC method development using C₁₈ inertsil column and optimization of variables for ambrisentan (ABN) estimation in bulk and formulations. A two-level full factorial design assisted development of a visible spectroscopic method based on the principle of oxidation and coupling reaction of ABN with 3-methyl-2-benzothiazolinone hydrazone (MBTH reagent) in presence of FeCl₃ to form bluish-green chromogen which is detectable in the visible range. second method estimates ABN in bulk and tablet dosage forms in the UV range using pH 7.4 phosphate buffer and the third one is a rapid, simple, stability indicating RP-HPLC method using a degassed mixture of orthophosphoric acid (OPA, 0.05M): acetonitrile (40:60) as mobile phase with water: acetonitrile (30:70) as diluent using PDA detector set at 264 nm for routine estimation of ABN in bulk and pharmaceutical dosage forms. Experimental design, data analysis and contour plots were developed using Minitab[®] 16.2.4 trial version (Minitab Inc). The percent assay of ABN detected by visible spectroscopy was 100.58±101.37% with RSD value 0.38%, by UV spectroscopy it was 99.1±99.8% with 0.5% RSD value and by RP-HPLC it was found 100.9%. The values of method validation parameters of three methodologies were found within the acceptance limits. The three methodologies could be regarded as specific, accurate, precise, with significant stability and robustness for routine estimation of ABN in bulk and tablet dosage forms.

Keywords: Ambrisentan, Antihypertensive, Experimental design, RP-HPLC, Spectrophotometric, Validation

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1. Introduction

Analytical method development is an integral part of preformulation and formulation development research. With the advancements in pharmaceutical researches, development of both small and large scale pharmaceutical industries worldwide, the number of drugs and drug formulations are increasing in the market day by day which in many cases may be new entities or partial modifications of the existing drugs or novel dosage forms. The assay procedure of such drugs or formulations may not be official in pharmacopeias. Hence, there arises dire need to develop rapid, simple, sensitive, specific, cost effective, validated and stability indicating analytical methods for the routine analysis of drugs. Analytical

method development is an imperative necessity since the design of the drug delivery system is related to it. Moreover drug analysis is also necessary in various steps of formulation design and dissolution studies [1-5]. Sophisticated hyphenated techniques like LC-MS, LC-ESI-MS/MS though introduced into analytical research but relatively expensive, necessitates complicated sample preparation steps, time consuming and obviously not preferable to small scale industries for routine and rapid analysis [6,7]. UV-visible spectrophotometric method is one of the earliest, yet easy, sensitive, relatively cost effective method applied for drug estimation in both small and large scale pharmaceutical industries. Properly validated, stability indicating HPLC methods if be precise, rapid and simple in operation, is of immense utility in routine quality control [2-6].

Ambrisentan (ABN), chemically, (2S)-2-(4,6-dimethyl pyrimidin-2-ylloxy) -3-methoxy-3, 3-diphenyl propionic acid, an orally active antihypertensive drug used in the treatment of pulmonary arterial hypertension to improve exercise capacity and delay clinical worsening; is a non-peptide, highly selective endothelin-1 type A receptor antagonist. Endothelin (ET) is a peptide which elevates blood pressure by constriction of blood vessels. There are two classes of ET receptors namely, type A and type B. The binding of ET to type A receptors causes vasoconstriction while the binding to those of type B causes vasodilatation. ABN has high affinity to ET-type A receptor, thereby blocking its effect and decreasing blood pressure in lungs. The thickening of blood vessels in the lungs and heart is also inhibited by ABN [4,5,6].

To the best of our knowledge estimation of ABN is not official in any of the Pharmacopeias. Literature survey showed LC-ESI-MS/MS [6], enantio-selective liquid chromatography [7], spectrophotometry [8,9,10] and UV-HPLC methods for the estimation of ABN [11,12,17,18,19,20,21]. Considering the clinical significance of ABN, there is a need to develop simple, rapid, cost effective, validated analytical techniques for its routine estimation in formulations. The current research focuses to develop three methodologies for ABN quantification, one of which is based on oxidation of ABN followed by coupling with 3-methyl-2-benzothiazolinone hydrazone (MBTH) in presence of FeCl₃ to form bluish green color chromogen, detectable in visible spectroscopic range. The second method estimates ABN in bulk and tablet dosage forms in the UV range using pH 7.4 phosphate buffer and the third method is a rapid, simple, stability indicating RP-HPLC method for routine estimation of ABN in bulk and pharmaceutical dosage forms.

Developing and optimizing a new analytical method is a complex procedure necessitating simultaneous determination of several method parameters. Traditional trial-error approach is not only time consuming but provides an apparent optimization method. Information relating to factors influencing process output measures or product quality are not available. The current research utilized experimental design approach for evaluating the significance of the studied factors and optimizing the visible spectroscopic and chromatographic conditions [13,16].

2. Materials and Methods

2.1. Chemicals and Reagents

Pure ambrisentan was gratis sample from Cystron Laboratories, Vijayawada, India. HPLC grade acetonitrile, methanol and water were purchased from Merck, India. Sodium carbonate, HCl, orthophosphoric acid, ferric chloride, triethyl amine and other chemicals are of AR grade from Merck, India.

2.2. Instrumentation

Liquid chromatographic system (Waters, with EMPOWER2[®] software using PDA detector, India), UV-visible spectrophotometer (Thermo Scientific, Aquamate Plus, India), Electronic balance (Shimadzu, Japan),

Sonicator (Cyber labs, India), pH meter (Datla instruments, DI-45, India) and Hot Plate (Shital Scientific Industries, India) were employed in the study.

2.3. Software

Experimental design, data analysis and contour plots were developed using Minitab[®] 16.2.4 trial version (Minitab Inc).

2.4. Estimation of ABN by visible spectroscopy

The method is based on the principle of oxidation and coupling reaction of ABN with 3-methyl-2-benzothiazolinone hydrazone (MBTH reagent) in presence of FeCl₃ to form bluish-green chromogen which is detectable in the visible range. The proposed reaction scheme is presented in Figure 1.

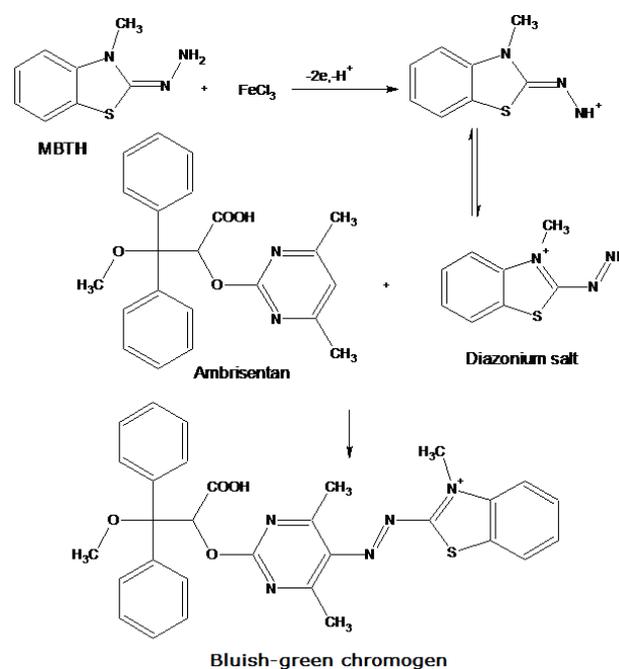


Figure 1. Proposed reaction scheme showing formation of bluish-green chromogen

2.4.1. Experimental Design for Optimization

A two-level full factorial design was employed to evaluate the effect of four independent factors, viz. MBTH reagent concentration (X₁), volume of MBTH reagent (X₂), FeCl₃ concentration (X₃) and Volume of FeCl₃ solution (X₄) on absorbance Y. The table matrix of the two-level full factorial design and experimental runs is provided in Table 1. From the resultant design matrix it was observed that maximum absorbance (Y) was shown with 2% MBTH and 2% FeCl₃ each of 2 mL and 1.5–2 mL respectively. Thus in the optimized method, 10 mg of ABN was dissolved in 10 mL methanol to get a concentration of 1000 µg/mL (standard stock solution) and further diluted with the same solvent to get a concentration of 10 µg/mL. This solution was scanned in the range of 400–800 nm against blank and 625 nm was selected as the λ_{max} for analysis of ABN by MBTH. To each flask, 1.5–2 mL of aqueous FeCl₃ solution (2% in distilled water) and 2 mL of MBTH reagent (2% in

distilled water) were added. Volumes were adjusted in each tube with distilled water. The absorbance of bluish green colored chromogen was measured at 625 nm against the blank. Different aliquots of ABN were prepared in 10 mL volumetric flasks in the concentrations of 10–30 µg/mL. The amount of ABN present in the sample solution was computed from its calibration curve [14].

2.4.2. Validation of the Method

The above method was validated as per ICH guidelines in terms of linearity, accuracy, precision, robustness, LOD and LOQ [22]. The linearity range for the estimation of ABN by MBTH was determined by preparing aliquots in the concentration range of 10–30 µg/mL and absorbances measured at 625 nm. Calibration curves (concentrations vs absorbance) were plotted and R² value not less than 0.99 was regarded as acceptance criterion.

Accuracy of the proposed method was ascertained by recovery studies using analyte recovery method and percent recovery for ABN in the range of 98.8–99.6% were set as the acceptance criterion. The precision was studied by intra-day variations in the test method of ABN and expressed as percent relative standard deviation (RSD) where these values should not be greater than 2%. The LOD and LOQ parameters were determined from the calibration curves basing on the formulae: $LOD = 3.3 \sigma / S$ and $LOQ = 10 \sigma / S$, where S is slope of calibration curve and σ is standard deviation of y-intercepts of calibration graph.

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in the process parameters. As a measure of robustness of the method, intentional alterations were made in the absorbance maximum to evaluate the impact of the method. A 20 µg/mL of ABN was prepared by above procedure and the absorbance was observed at 627 and 623 nm.

2.4.3. Assay of ABN

Accurately weighed 10 tablets (10 mg) were pooled and powdered of which 10 mg equivalent of ABN was transferred into 10 mL volumetric flask, dissolved in methanol, sonicated, filtered through 0.45 µm filter (Millipore®), and properly diluted to get a solution concentration of 100 µg/mL. The absorbance of ABN sample solution at 20 µg/mL concentration was measured at 625 nm against blank and the percentage assay was calculated using the formula:

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label claim}} \times 100$$

where, AT is absorbance of test, WS= wt. of standard sample, WT= wt. of test sample, AS is absorbance of standard, DS is dilution factor of standard, DT is dilution factor of test and P is potency of standard.

2.5. Estimation of ABN by UV Spectroscopy

The 0.1 M pH 7.4 buffer solution was prepared by dissolving 2.62 g of monobasic sodium phosphate and 11.5 g of anhydrous dibasic sodium phosphate in 1000 mL of water. Stock solution was prepared by dissolving 10 mg of ABN in pH 7.4 phosphate buffer and diluted to get a concentration of 10 µg/mL. This solution was scanned in

200–400 nm range against blank and 262.5 nm was selected as λ_{max} for analysis of ABN. The standard calibration curve was constructed with the standard stock solution in the concentration range of 10–50 µg/mL and absorbances were measured at 262.5 nm. The UV method developed was validated in terms of linearity, accuracy, precision, LOD, LOQ and robustness as discussed above.

For preparation of ABN sample solution, accurately weighed 10 tablets of ABN were powdered of which 10 mg equivalent of the drug was transferred into 10 mL volumetric flask, dissolved in 7 mL of phosphate buffer, sonicated well, filtered through 0.45 µm filter (Millipore®) and diluted to get a concentration of 100 µg/mL. The absorbance of ABN sample (20 µg/mL) was measured at 262.5 nm against blank and the percentage assay was calculated using the above formula.

2.6. Estimation of ABN by RP–HPLC

2.6.1. Experimental Design and Optimization of Mobile Phase

Before proceeding to the optimized RP–HPLC chromatographic conditions for ABN estimation, numerous trials were conducted with different mobile phase compositions. Amongst fifteen preliminary trials, injection volume (10 µL) and flow rate (1 mL/min) were kept constant. The absorbances were measured between 260–264 nm wavelength and trial runs were performed using different mobile phase compositions. Using triethylamine (TEA) as mobile phase with pH adjusted by 0.05M orthophosphoric acid (OPA), a very broad peak was obtained. The same result was reported when methanol was used as diluents, when water: methanol (80:20) was used as diluents the peak retention time became unduly delayed. On changing the mobile phase composition to TEA: OPA: acetonitrile (ACN) with diluents of varying compositions like water: acetonitrile (50:50) and methanol: water (80:20), the peaks were not within symmetry limits with a noisy baseline indicating instability of the drug in the mobile phase. A two-level full factorial design [13,14,15,16] was employed to evaluate the effect of three independent factors, viz. wavelength (X1), flow rate (X2) and OPA percentage in mobile phase (X3) on retention time (Y1) and USP tailing factor (Y2). The table matrix of the two-level full factorial design and experimental runs is provided in Table 2.

The optimized chromatographic conditions for ABN estimation were obtained with isocratic separation mode in a C₁₈ inertsil column (250 mm × 4.6 mm, 5 µm) using a degassed mixture of orthophosphoric acid (OPA): acetonitrile (40:60) as mobile phase with water: acetonitrile (30:70) as diluent, injection volume (20 µL), flow rate (1.2 mL/min) and run time (10 min) with PDA (Photo Diode Array) detector set at 264 nm.

The working standard and sample solutions of ABN were prepared by dissolving 65 mg ABN in diluent (water: acetonitrile, 30:70), sonicated and volume adjusted with diluent. Further dilutions from 5–50 µg/mL concentration range were prepared from the standard and sample solutions, and the volume was adjusted in each case with mobile phase.

2.6.2. System Suitability

The ABN standard solution was injected into the HPLC system six times to evaluate the system suitability parameters from the standard chromatograms obtained by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from six replicate injections. The limit of RSD and tailing factor were set below 2.0%. The theoretical plate count was considered if less than 2000.

2.6.3. Validation

The proposed RP-HPLC method was validated as per ICH guidelines [22]. Accuracy of the proposed method was ascertained by recovery studies using analyte recovery method, where the drug substance was spiked with placebo in concentrations of 50, 100 and 150% and the acceptable criteria of percent recovery for ABN were set as 98–102%. Precision was studied in terms of repeatability (system precision), where 50 µg/mL of sample was injected for six times into the HPLC system as per test procedure. For method precision, from sample and stock solution, six replicates of standard and sample of 50 µg/mL were prepared and injected into the HPLC system and % RSD was calculated. Intermediate precision study or ruggedness of experimentation was carried out by different analyst, on different instrument and on different days. From the sample and stock solutions, six replicates of 50 µg/mL were prepared and injected into the HPLC system and % RSD was calculated. The LOD and LOQ parameters were determined from the calibration curves basing on the formulae above.

As a measure of robustness of the method, deliberate alterations in the absorbance maximum, flow rate and mobile phase composition were made to evaluate the impact of the method. The λ_{\max} was changed (± 5 nm) from 264 nm to 269 and 259 nm. The flow rate was increased from original value (1.2 mL/min) to 1.4 mL/min and decreased to 0.8 mL/min. The ratio of mobile phase composition was also altered ($\pm 10\%$) from 40:60 to 30:70 and 50:50. The tailing factor of ABN standard, RSD of asymmetry and retention time of ABN standard should not be more than 2% due to the intentional alterations of the said parameters.

2.6.4. Interference Studies

For studying blank and placebo interference, diluents and sample of placebo were injected in to the system with the objective that they should not show any peak at the retention time of analyte peak.

2.6.5. Degradation Studies

Chemical degradation studies such as; acidic, alkaline, oxidative, reductive, thermal, photolytic and humidity were conducted under stress conditions. From the sample stock solution, aliquots of 5 mL was transferred into 20 mL volumetric flask. To this, a 2 mL of 5% either HCl or NaOH was added for acid or alkali degradation studies respectively. In oxidation study, 2 mL of H₂O₂ was added, while in reduction study a 2 mL of sodium bisulphite was added. A small volume of diluent was added in all solutions, heated at 80°C on water bath for 20 min and cooled. The solutions were neutralized if necessary and the volume was adjusted with diluent. In case of thermal degradation studies the diluent was added to adjust the

volume in the flask and the solution was heated at 105°C for 24 h, whereas in photolytic degradation, the solution was kept under sunlight for 24 h. During humidity stress investigation, the solution was subjected to 90% RH at 25°C for 24 h. The above solutions were filtered through 0.45 µm nylon filter (Millipore®) by discarding first 4 mL of solution. The blank solutions were prepared in the same manner without using samples.

2.6.6. Bench Top Solution Stability of Standard and Sample

To evaluate the solution stability, standard solution was prepared in single and samples were prepared in duplicate and kept on bench top. Solutions were analyzed at 0, 4, 8, 12 and 24 h. The differences of ABN content (%) for bench top samples were calculated from the results as follows: Difference of assay (%) = % of assay at each time interval (h) – % assay at initial time (0 h).

2.6.7. Assay of ABN in Bulk and Dosage Forms

Prior to the injection of drug (ABN) solutions, the column was equilibrated for at least 30 min with the mobile phase with a flow rate of 1.2 mL/min. Then 20 µL of standard and sample solutions were injected for six and two times respectively and the drug content was determined. For the estimation of ABN in dosage forms, accurately weighed 10 tablets of ABN were powdered. A 10 mg equivalent of the drug was transferred into 10 mL volumetric flask, the diluent was added, sonicated well, filtered through 0.45 µm filter (Millipore®) and suitably diluted and injected into HPLC system and the drug content was determined using standard curve.

Table 1. Two Level Full Factorial Design and Response Values for Visible Spectroscopy

SO	RO	PT	B	X1	X2	X3	X4	Y
3	1	2	1	0	2	1	1	0.119
25	2	0	1	1	1	1	1	0.757
2	3	2	1	2	0	1	1	0.234
15	4	2	1	1	0	2	1	0.384
9	5	2	1	0	1	1	0	0.211
12	6	2	1	2	1	1	2	0.627
16	7	2	1	1	2	2	1	0.675
17	8	2	1	0	1	0	1	0.201
6	9	2	1	1	1	2	0	0.321
19	10	2	1	0	1	2	1	0.119
24	11	2	1	1	2	1	2	0.701
4	12	2	1	2	2	1	1	0.715
26	13	0	1	1	1	1	1	0.757
23	14	2	1	1	0	1	2	0.118
27	15	0	1	1	1	1	1	0.757
13	16	2	1	1	0	0	1	0.113
14	17	2	1	1	2	0	1	0.451
1	18	2	1	0	0	1	1	0.381
22	19	2	1	1	2	1	0	0.294
7	20	2	1	1	1	0	2	0.632
8	21	2	1	1	1	2	2	0.699
5	22	2	1	1	1	0	0	0.471
10	23	2	1	2	1	1	0	0.511
20	24	2	1	2	1	2	1	0.712
21	25	2	1	1	0	1	0	0.11
18	26	2	1	2	1	0	1	0.213
11	27	2	1	0	1	1	2	0.114

SO = standard order, RO = run order, PT = Pt type, B = blocks, X1 = MBTH reagent conc., X2 = volume of MBTH reagent, X3 = FeCl₃ concentration, X4 = FeCl₃ volume, Y = Absorbance

3. Results

3.1. Visible and UV Spectroscopy

The matrix of the two-level full factorial design and experimental runs for visible spectroscopy is provided in Table 1. The contour plots of absorbance vs. FeCl₃ and MBTH concentration and volume are presented in Figure 2. Data of the calibration curve of ABN by visible and UV

spectroscopy are summarized in Table 2. The percent assay of ABN by visible spectroscopy was 100.58–101.37% with 0.38% RSD and that detected by UV spectroscopy was found 99.1–99.8% with 0.5% RSD. Results of visible and UV spectroscopic method validations in terms of accuracy (recovery studies), precision, specificity, LOD and LOQ and robustness are provided in Table 3.

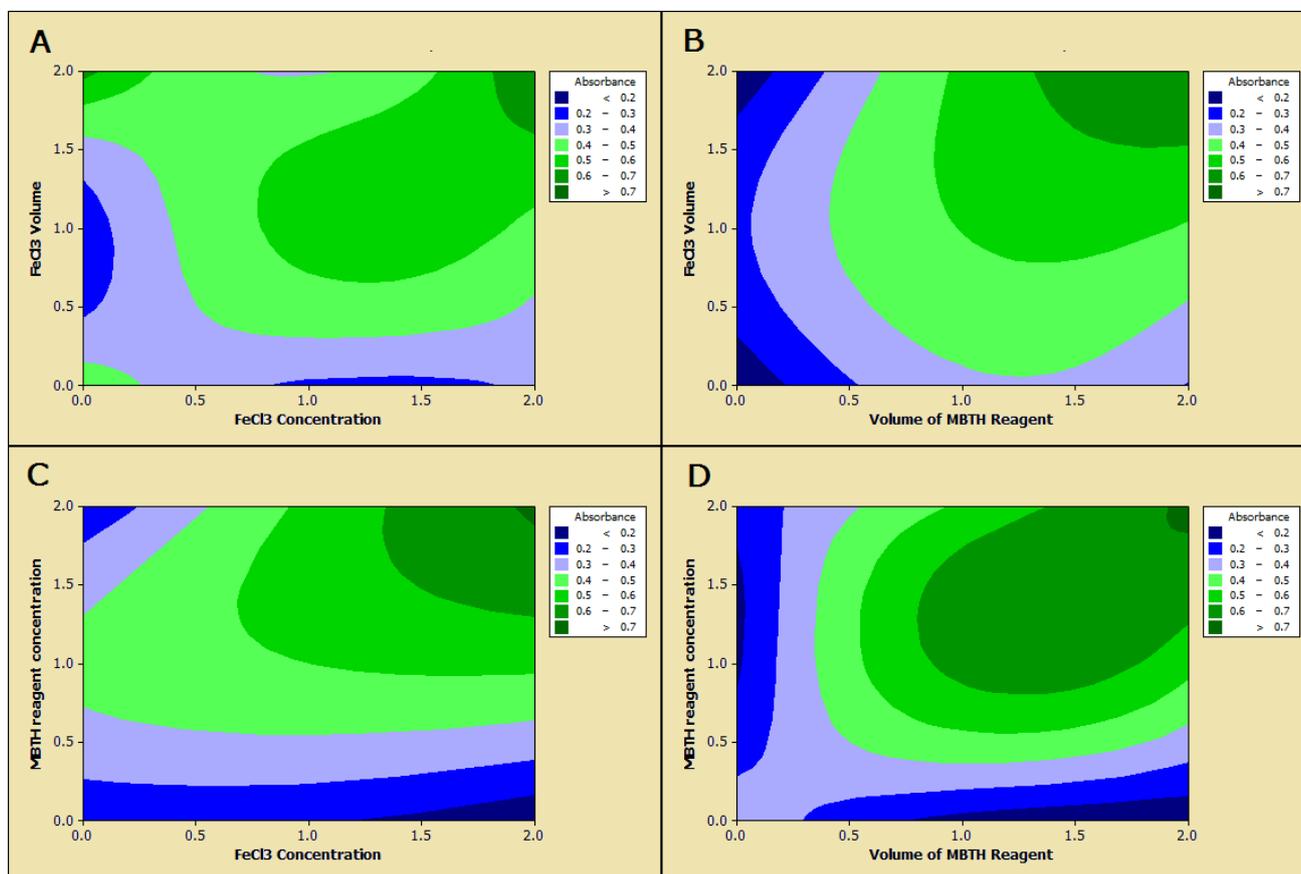


Figure 2. Contour plots of (A) absorbance vs FeCl₃ concentration and volume, (B) absorbance vs. FeCl₃ and MBTH volume, (C) absorbance vs. MBTH and FeCl₃ concentration, and (D) absorbance vs. MBTH concentration and volume

Table 2. Data of the Calibration Curve of Ambrisentan by Visible and UV Spectroscopy

Parameters	Visible method	UV method
<i>95% confidence intervals</i>		
Slope	0.02407 to 0.02765	0.01075 to 0.01237
y-intercept	-0.05349 to 0.02229	-0.01815 to 0.03575
x-intercept	-0.922 to 1.943	-3.304 to 1.476
<i>Goodness of Fit</i>		
R ²	0.9986	0.9985
P value	< 0.0001	< 0.0001
Equation	y = 0.02586x - 0.01560	y = 0.01156x + 0.008800
<i>Best fit values</i>		
Slope	0.02586 ± 0.00056	0.01156 ± 0.00025
y-intercept	-0.01560 ± 0.01191	0.008800 ± 0.008469
x-intercept	0.6032	-0.7612

Table 3. Validation Studies of Ambrisentan by Visible and UV Spectroscopy (n=6)

Parameters	Recommended limits	Visible method	UV method
Specificity	No interferences	^c	^c
Precision ^a	NMT ^b 2.00	1.04	0.871
Accuracy, % (^d)	98–102 (2.00)	98.8–99.6 (0.439)	100.3–100.9 (0.527)
LOD and LOQ	S:N ratio should be more than 3:1	2.7 and 8.3	1.2 and 3.6
Robustness ^a , (wave length, nm)	NMT ^b 2.00	1.74 (627) 1.02 (623)	0.236 (260.5) 0.229 (264.5)

^aPercentage relative standard deviation, ^bNot more than, ^cSpecific nature of method, no interferences found

3.2. RP-HPLC Method

The response values of experimental runs of two-level full factorial design on HPLC method are given in Table 4. The different contour plots of the RP-HPLC experimental design are shown in Figure 3. As regards method validation, the linearity of the method was demonstrated over the concentration range of 10–300 $\mu\text{g/mL}$ with R^2 value not less than 0.99 and RSD of peak areas of the solution not more than 2.0%. From the assay calculations, the assay of ABN tablets was found 100.9% and 0.35% RSD. There was no interference due to blank and placebo at the retention time of the analyte (Figure 4). The recovery of the spiked drug was found 100.7% with 0.23% RSD which falls within the acceptance criterion. Regarding precision, the system precision or repeatability of the method showed 0.632% RSD for the peak areas. The label claim for method precision was found 100.6% with 0.23% RSD. For intermediate precision or ruggedness of the method, 0.23% RSD was found for the peak area. Thus all parameters of precision studies were

less than 2.0. LOD and LOQ values were calculated as 1.967 and 5.961 respectively.

Table 4. Two Level Full Factorial Design and Response Values for RP-HPLC

SO	RO	PT	B	W	FR	OPA	RT	TF
9	1	2	1	264	1	30	6.2	1.14
12	2	2	1	264	1.4	50	3.5	1.18
5	3	2	1	259	1.2	30	6.2	1.13
8	4	2	1	269	1.2	50	3.5	1.19
11	5	2	1	264	1	50	3.5	1.19
1	6	2	1	259	1	40	4.4	1.15
7	7	2	1	259	1.2	50	3.5	1.18
13	8	0	1	264	1.2	40	4.4	1.15
6	9	2	1	269	1.2	30	6.2	1.14
15	10	0	1	264	1.2	40	4.4	1.14
14	11	0	1	264	1.2	40	4.4	1.15
2	12	2	1	269	1	40	4.4	1.14
10	13	2	1	264	1.4	30	6.2	1.13
3	14	2	1	259	1.4	40	4.4	1.15
4	15	2	1	269	1.4	40	4.4	1.14

SO = standard order, RO = run order, PT = Pt type, B = blocks, W = wave length (nm), Flow rate (mL/min), OPA = OPA % in mobile phase, RT = retention time, TF = USP tailing factor.

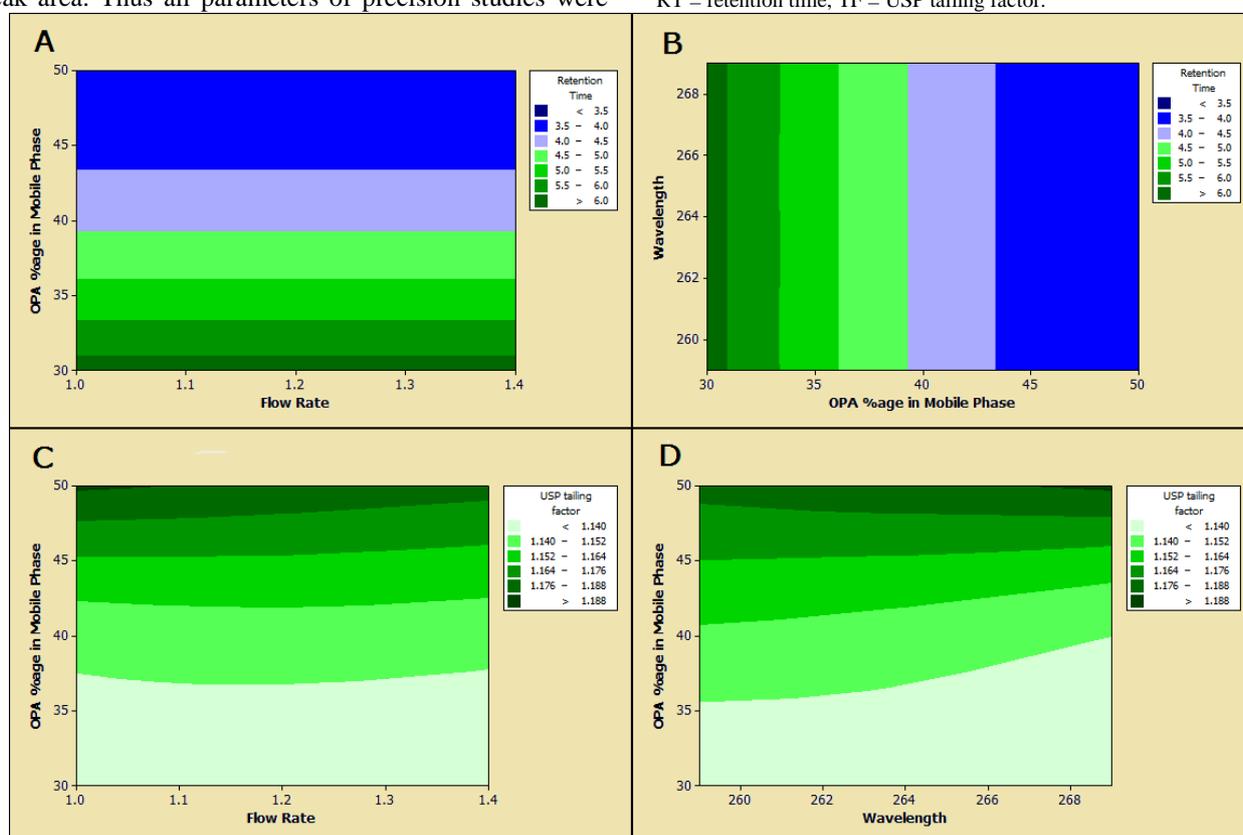


Figure 3. Contour plots of (A) retention time vs. OPA concentration and flow rate, (B) retention time vs. wavelength and OPA concentration, (C) USP tailing factor vs. OPA concentration and flow rate, and (D) USP tailing factor vs. OPA concentration and wavelength

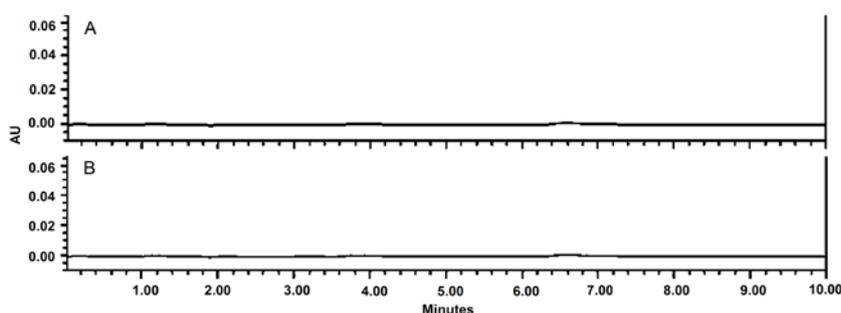


Figure 4. Typical chromatograms of (A) blank and (B) placebo

Results of robustness of the method as determined by deliberate alterations in the ratios of mobile phase composition, flow rate and wavelength range are presented in Table 5. The results of forced degradation studies are presented in Table 6. Results of bench top solution stability studies are provided in Table 7 and Figure 5.

Table 5. Robustness of the Developed RP-HPLC method (n=6)

Parameters	Peak area (%RSD) ^a
<i>Flow rate (mL/min)</i>	
1.2 ^b	0.082
1.4 ^c	0.086
0.8 ^d	0.199
<i>Wave length (nm)</i>	
264 ^b	0.082
269 ^c	0.184
259 ^d	0.211
<i>Mobile phase^e</i>	
40:60 ^b	0.082
50:50 ^f	0.870
30:70 ^f	0.420

^aPercentage relative standard deviation, ^bActual value, ^cIncreased

^dDecreased, ^eOrthophosphoric acid:acetonitrile, ^fAltered ratio

Table 6. Forced Degradation Studies of Ambrisentan by Developed RP-HPLC Method (n=6)^a

Type	RT	A	PC	TF	Deg	PA	PT
Acid	6.203	180271	7658	1.097	9.9	0.327	1.331
Alkali	6.199	49127	7295	1.105	17.5	0.219	1.209
Peroxide	6.212	587417	7565	1.032	25.7	0.356	1.190
Reduction	6.206	320109	7794	1.110	17.0	0.232	1.222
Thermal	6.199	220355	7746	1.096	9.3	0.368	1.384
Photolytic	6.200	807180	7672	1.105	6.2	0.201	1.133
Humidity	6.207	807291	7857	1.098	6.8	0.219	1.130

Percentage relative, RT = retention time (min), A = peak area, PC = USP plate count, TF = USP tailing factor, Deg = % degradation, PA = purity angle, PT = purity threshold. ^aAll values comply with limits.

Table 7. Bench Top Solution Stability Studies of Ambrisentan by Developed RP-HPLC Method (n=6)

Time (h)	Label claim (%)	% RSD ^a
0	99.5	0.5
4	100.3	0.8
8	101.2	1.7
12	101.4	1.9
24	101.5	2.0

^aPercentage relative standard deviation

4. Discussion

A simple, rapid and properly validated UV spectroscopic method was developed for estimation of ABN in bulk and tablet dosage forms. Earlier work reported a single UV method [10] for ABN quantifications using acetonitrile as the diluent, whereas the current method employed pH 7.4 phosphate buffer as the solvent. Two methods reported ABN estimation in visible range by forming colored ion-pair complexes of ABN with methylene blue and safranin [8] and by charge transfer reaction of ABN with dichloro benzoquinone derivatives [9]. But the present work applied principle of oxidation and coupling reaction of ABN with MBTH reagent in presence of FeCl₃ to form bluish-green chromogen which is detectable in the visible range. The two reported RP-HPLC methods [11,12] used mobile phase in gradient mode but we developed an optimized method using isocratic separation mode.

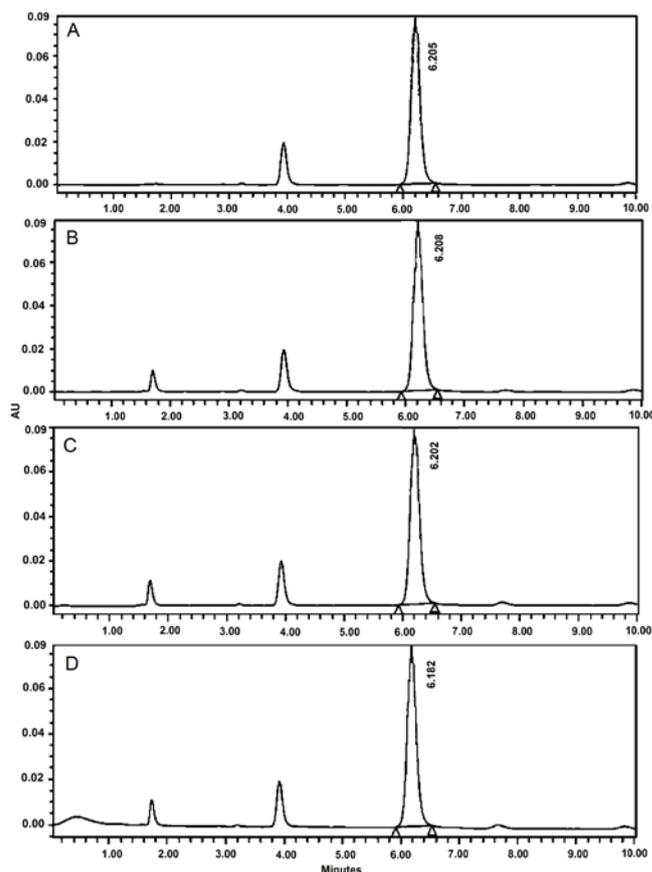


Figure 5. Chromatograms of solution stability at (A) initial time, (B) 6 h, (C) 12 h and (D) 24 h

A two-level full factorial design was utilized for proper modeling and optimization of process parameters in visible spectroscopy and RP-HPLC methods. With the help of contour plots optimum values of selected factors were identified (Figure 2 and Figure 3). There was a good match between the predicted values and experimental values. A considerable perception was obtained about the effect of variables on the selected response Y. The results of visible and UV spectroscopy showed high linearity correlation coefficient ($R^2=0.99$). The percent assay of ABN was determined using both visible and UV spectroscopic methods were found appreciable. Since there was no interference, both the methods can be said to be specific. High values of percent recovery in both studies, RSD values of precision and robustness, values of LOD and LOQ, all being within the specified acceptance limits (Table 4). The two spectroscopic methods were found specific, accurate, and precise with enough robustness and could be applied in the routine estimation of ABN.

The RP-HPLC method developed showed high percent purity of ABN in tablet formulations. Results of method validation studies in terms of linearity, accuracy, precision, system suitability, LOD and LOQ were within the specified acceptance limits. The developed RP-HPLC method was said to have qualified the validation parameters. Since there was no interference of blank and placebo during the retention time of the analyte, the developed method possessed high specificity. The RSD values of peak areas due to deliberate alterations in flow rate, wavelength and mobile phase compositions being within acceptance limits (Table 5), signifies the high

robustness of the developed method. Considering the results of forced degradation studies (Table 6) in any study media (acid, alkali, peroxide, reducing) and forced environmental conditions (photolytic, thermal or excess humidity), the developed method conforms to the standard limits. Similar is the case with bench top stability studies (Table 7) where the deviation did not exceed the limits of 2.0% at 24 h, suggesting that the developed RP-HPLC method is a stability indicating and validated one, thus contributing another new analytical method to the quality control tests of ABN.

5. Conclusions

ABN is an orally active antihypertensive drug used in the treatment of pulmonary atrial hypertension and no official method is reported in any of the pharmacopeias for its routine estimation. The reported hyphenated techniques involve expenditure, time and critical methodologies. Experimental design approach was employed for modeling and optimization of process parameters in visible spectroscopy and RP-HPLC methods. The current research was successful in developing three analytical techniques with the aid of HPLC, UV and visible spectroscopy which were found simple, accurate, precise, robust and stable. The developed methods could be employed for routine estimation of ABN in bulk and pharmaceutical formulations. The two-level full factorial design approach was effectively used to develop and optimize the spectrophotometric and RP-HPLC methods for ABN estimation.

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