A Stability-Indicating Reversed-Phase High-Performance Liquid Chromatography Method for Ambrisentan: An Endothelin Receptor Antagonist

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A stability-indicating reversed-phase high-performance liquid chromatography (HPLC) method was developed for the determination of ambrisentan, a drug used in the treatment of pulmonary hypertension. The desired chromatographic separation was achieved on a Kromasil C18 column (250 × 4.6 mm, 5 μm) using the mobile phase acetonitrile–ammonium formate (pH 3.0; 0.02 M) in gradient mode. The flow rate was set at 1.0 mL/min, and chromatograms were extracted at 262 nm using a photodiode array detector. The method was successfully validated in accordance to International Conference on Harmonization (ICH) guidelines acceptance criteria for linearity, accuracy, precision, robustness and forced degradation studies, which further proved the stability-indicating power. Linearity of ambrisentan peak area responses was demonstrated within the concentration range of 25–200 μg/mL. The limits of detection and quantitation were 0.2 and 0.6 μg/mL, respectively. Forced degradation studies were performed on ambrisentan bulk drug samples as per ICH guidelines to demonstrate the stability-indicating power of the HPLC method. Significant degradation was observed during acidic hydrolysis, neutral hydrolysis and oxidative stress. The degradation products were well resolved from primary peak of ambrisentan, indicating that the method is specific and stability indicating. The newly developed method is applicable for assay determination of active pharmaceutical ingredient.

Introduction

Ambrisentan, an orally active, highly selective antagonist of the endothelin-1 type A receptor, is indicated for the treatment of pulmonary arterial hypertension (1). Chemically, it is described as (2S)-2-[(4, 6-dimethylpyrimidin-2-yl)oxy]-3-methoxy-3,3-diphenylpropanoic acid (Figure 1). In vitro studies with ambrisentan have demonstrated that it is a potent and selective inhibitor of the endothelin-1 type A receptor. It is an approved drug for the treatment of pulmonary hypertension in USA and Europe. It improves exercise capacity and delays clinical worsening in patients with WHO Class II or Class III symptoms. $S$-Enantiomer of ambrisentan is active, and separation of enantiomers has been reported by chiral chromatography (2). Quantification of ambrisentan by high-performance liquid chromatography-positive ion electrospray tandem mass spectrometry [LC-ESI-MS/MS] method (3) was reported for biological samples. Few methods reported in the literature dealt with the analysis of ambrisentan using spectroscopy (4, 5). So far, to our present knowledge, no stability-indicating assay method for ambrisentan is available in the literature. Keeping this in view, stability studies for ambrisentan was carried out by forcing the drug under variety of conditions such as heat, light, oxidation and hydrolysis (acid, base and neutral). The developed HPLC assay method was validated as per ICH guidelines (6).

Experimental

Materials and reagents

The drug $S$-isomer of ambrisentan, >99.5% purity was received as a gift sample from MSN Laboratories Ltd (Hyderabad, India). The following excipients were purchased from commercial sources and used as such: magnesium stearate and lactose monohydrate (SD Fine-Chem Limited, Mumbai, India), microcrysteline cellulose (Blanver, USA), croscarmellose (FMC India Private Ltd, Bangalore, India). HPLC-grade acetonitrile (ACN) was purchased from SD Fine-Chem Limited. Ammonium formate was purchased from SD Fine-Chem Limited. High-purity water was obtained from MilliPak Millipore water purification system (Milli-Q, USA). All other reagents are of analytical grade.

Instrumentation

The HPLC system used for chromatographic method development, forced degradation studies and validation was Waters e2695 separation module with a 2998 model diode array detector. Separation was achieved on a Kromasil C18 column (250 × 4.6 mm, 5 μm). Mobile phase consisting of mixture of A: 0.02 M aqueous ammonium formate, with pH adjusted to 3.0 with formic acid and B: ACN was used. The timed gradient program: 7′ (min)/%B: 0/20, 5/40, 20/85, 30/20 with a flow rate of 1 mL/min were employed. Column temperature was maintained at 25°C and detection was carried out at 262 nm. Injection volume was 20 μL.

Methods

Preparation of standard solutions

A stock solution of ambrisentan (1 mg/mL) was prepared by dissolving 25 mg in minimum of ACN, sonicated for 10 min and then the volume was made up to 25 mL in a volumetric flask. This solution was diluted further with ACN to get a concentration of 200 μg/mL.

Forced degradation studies

Forced degradation studies/stress studies were performed on drug molecules to identify the likely degradation products which in turn help to elucidate the degradation pathway and intrinsic stability of the molecule (7). Stress studies were also indicative of the specificity of the developed method. Degradation of ambrisentan was attempted using heat, light, oxidation and hydrolysis (acid, base and neutral). All the forced degradation studies were carried out at an initial drug concentration of 1 mg/mL and the samples were diluted to the concentration of 200 μg/mL before injection using ACN as diluent. Acidic stress
was performed in methanolic HCl (1 N) at 80°C for 2 h. Degradation study in basic solution was done in aqueous NaOH (2 N) at 80°C for 4 h. Neutral hydrolysis was carried out using water at 80°C for 40 min. Oxidative stress study was carried out using methanolic H$_2$O$_2$ (3%) for 48 h. Photodegradation studies were carried out as per the ICH Q1B guideline where the sample was exposed to an overall illumination of 1.2 million lux h and a cumulated ultraviolet energy of 200 Wh/m$^2$ (8). Thermal stress was induced by exposing the sample to dry heat at 80°C for 7 days. Stressed samples were subjected to analysis after suitable dilution (200 μg/mL) to evaluate the ability of the developed method to separate ambrisentan from its degradation products.

**Procedure for acidic degradation**

One milligram per milliliter solution of ambrisentan in methano-lic HCl (1 N) was subjected to reflux at a temperature of 80°C for 2 h. At the end of 2 h, the solution in a round-bottomed flask was cooled to room temperature and was neutralized to pH 7. This neutralized solution was diluted appropriately to get a final concentration of 200 μg/mL using ACN.

**Procedure for alkaline degradation**

One milligram per milliliter solution of the drug in aqueous NaOH (2 N) in a round-bottomed flask was refluxed at 80°C for 4 h. The flask was cooled and the contents were neutralized to pH 7. The solution was diluted appropriately to a concentration of 200 μg/mL using ACN.

**Procedure for neutral degradation**

A suspension of drug in water (1 mg/mL) was refluxed at 80°C for 40 min. This suspension was cooled and solubilized using ACN before injection.

**Procedure for oxidative degradation**

Ten milligrams of drug were transferred into a 10-mL volumetric flask to which 3 mL of 30% H$_2$O$_2$ was added and volume was made to 10 mL with methanol. This flask was wrapped in aluminum foil and kept in dark for 48 h. Similarly a blank solution was prepared and stored in dark for 48 h. Solution was diluted appropriately to a concentration of 200 μg/mL using ACN before injection.

**Procedure for thermal degradation**

Twenty-five milligrams of ambrisentan were transferred into a borosilicate petri plate and spread uniformly before placing in an oven at a temperature of 80°C for 7 days.

**Procedure for photolytic degradation**

Stability of the drug on exposure to both UV and fluorescent light sources was tested. Twenty-five milligrams of ambrisentan were taken in a borosilicate petri plate and placed in the stability chamber for an overall illumination of 1.2 million lux h and a cumulated ultraviolet energy of 200 Wh/m$^2$ with UV radiation in the range of 320–400 nm. Similarly, control was observed by wrapping an aluminum foil around the petri plate and kept in the stability chamber.

**Method validation**

**Specificity**

Specificity was estimated by spiking excipients such as magne-sium stearate, lactose monohydrate, microcrystalline cellulose and croscarmellose into a preweighed quantity of drug.

**Limit of detection and limit of quantification**

Limit of detection (LOD) and limit of quantification (LOQ) were obtained at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions of known concentrations of ambrisentan using Empower II software.

**Linearity**

Linearity depicts the ability of an analytical procedure to obtain test results, which are directly proportional to the concentration of the analyte in the sample within a certain range. It was established by injecting seven concentrations of ambrisentan, namely 25, 50, 75, 100, 125, 150 and 200 μg/mL. The peak area versus concentration was plotted, and the correlation coefficient of the calibration curve was calculated.

**Precision**

Precision of the analytical procedure was evaluated by the deter-mination of repeatability of the method (intraday precision) and intermediate precision (interday precision) of the sample solutions. Repeatability was calculated by assaying six samples of 100 μg/mL each during the same day, under the same experimental conditions. The intermediate precision was studied by comparing the results of the assay on three different days. The relative standard deviations (RSDs) of the peaks were calculated.

**Accuracy**

Accuracy was evaluated by applying the proposed method to the analysis of a mixture of the excipients with known amounts of the drug, to obtain solutions at concentrations of 80, 100 and 120 μg/mL, equivalent to 80, 100 and 120% of the nominal analytical concentration, respectively. The accuracy was calculated as the percentage of the drug recovered from the formulation matrix.

**Robustness**

Robustness of the method was established by studying the intentional variations in chromatographic conditions such as flow
rate, column temperature and mobile-phase pH. Each condition was varied separately and the effect of these changes on peak tailing and theoretical plates was observed.

**System suitability**
System suitability parameters were measured using three injections of a drug solution containing 100 μg/mL of ambrisentan to verify the system performance. RSDs of the retention time, tailing factor, number of theoretical plates and peak area were measured to test system suitability.

**Results**

**Method development and validation**

**Specificity and forced degradation**
Specificity is the ability of the method to discriminate the analyte from its potential degradation products. All the degradation products were well separated from the analyte peak which confirms the specificity of the developed method. Moreover, there were no interfering peaks of tablet excipients at the retention time of drug and degradation products as well, which also proves the specificity of the method. Figure 2 shows the chromatographic profiles of placebo spiked sample chromatogram, oxidation, hydrolysis (acid and neutral), standard and blank chromatogram. Results of forced degradation studies are summarized in Table 1. Ambrisentan were found to be stable under photolytic stress, thermal stress and alkaline stress conditions. Significant degradation was observed during acidic, neutral and oxidative stress conditions. The peak purity tool was employed to confirm the absence of other substances co-eluting with each other. In acid hydrolysis, the amount of drug degraded was \(\sim 50.26\%\) with six degradation products formed. A major degradation product constituting 39.97% was observed at the retention time \(\sim 24.4 \text{ min}\). In neutral hydrolysis, the amount of drug degraded was found to be \(\sim 59.28\%\) with two degradation products, one of them comprising 58.24% at \(\sim 24.4 \text{ min}\). Under oxidative stress conditions, a number of degradation products were formed constituting only 5% degradation. Drug was stable to alkaline hydrolysis when refluxed at 80°C for 4 h in 2 N NaOH. No significant degradation was observed when ambrisentan powder

![Figure 2](https://academic.oup.com/chromsci/article-abstract/52/8/894/277151)

(a) Placebo spiked sample chromatogram, (b) neutral hydrolysis, (c) oxidative degradation, (d) acid hydrolysis, (e) ambrisentan standard and (f) blank chromatogram.
and control samples were exposed to a cumulated ultraviolet energy of 200 Wh/m² in a photostability chamber. No decomposition was found on exposure of ambrisentan to dry heat at 80°C for 7 days in a hot air oven, depicting that it is thermo stable.

Limits of detection and quantification. LOD was 0.2 µg/mL for ambrisentan at a signal-to-noise ratio of 3:1 and the LOQ was estimated to be 0.6 µg/mL for ambrisentan at a signal-to-noise ratio of 10:1.

Linearity. Linearity of the method was established by least square regression analysis of the calibration curve. Method was proved to be linear in the concentration range of 25–200 µg/mL. The value of correlation coefficient calculated for ambrisentan was 0.9998, \( y = 19873.75x + 2963.26 \) was shown in Figure 3. The linearity was checked in the same concentration range on the consecutive days. There was an excellent correlation between the peak area and the concentration of the analyte.

Precision. The percentage relative standard deviation (%RSD) of ambrisentan for six consecutive injections of 100 µg/mL solution was well within 2%. Results of intra- and interday precision are presented in Table II. The %RSD for intraday precision was 1.04 and 0.65% for interday precision.

Accuracy. The data for accuracy were expressed in terms of percentage recovery of ambrisentan from placebo spiked samples. The results are summarized in Table III. The mean recovery was in the range of 100.75–101.34% which demonstrated that the method was accurate.

Robustness. Robustness of the method was checked by deliberately altering the conditions like flow rate, temperature and pH of the mobile phase. The effect of these changes on peak tailing and theoretical plates has been observed Table IV. There were no significant deviations observed.

### Discussion

The main target of the chromatographic method is to separate the degradation products generated during forced degradation from the analyte peak. Initial method development was attempted with ammonium acetate buffer (0.01 M) with pH 5.0 as solvent A and ACN as solvent B. This was not successful because it resulted in large variation in peak areas from injection to injection. This behavior is because of secondary equilibria on the analyte retention (9). At least a difference of one unit between mobile-phase pH and pKₐ of the analyte is preferable to maintain the analyte in a predominant single-ionization state. But at higher pH, carboxylic functional group in ambrisentan ionizes and gets solvated with the water molecules in the mobile phase.
phase which leads to abnormal retention behavior. Therefore, the mobile-phase pH less than the pK_a of ambrisentan were preferred. Since, the pK_a of ambrisentan is 4.0, ammonium formate buffer (0.02 M) with pH 3.0 adjusted with formic acid as solvent A and ACN as solvent B was employed. Kromasil column with C_{18} bonded phase, 5 μm particles with 100 Å pore size having dimensions of 250 × 4.6 mm at 1.0 mL/min flow rate was used. Initially, the method was developed in isocratic mode but later it was shifted to gradient mode to separate the degradants that were not eluting under isocratic mode. Hence, a timed gradient program: T(min)/%B: 0/20, 5/40, 20/85 and 30/20 with a flow rate of 1 mL/min is employed. Under these conditions, results were as follows: retention time of ambrisentan was ~15.8 min with a tailing factor of 1.33, number of theoretical plates (N) for ambrisentan peak was 86,218 and all the degradation products were well separated from the ambrisentan peak. Peak purity of ambrisentan was checked by using photodiode array detector with Empower II software, the purity angle was less than the purity threshold in all the stressed samples, thus depicting the homogeneity of the analyte peak.

Conclusion
A precise, accurate, specific, stability-indicating reversed-phase HPLC method for ambrisentan was developed. Behavior of ambrisentan under various stress conditions was studied. The developed method allows the separation of ambrisentan from its acidic, neutral and oxidative degradation products. Quality monitoring of ambrisentan in its bulk and stability samples can be achieved by employing the proposed method.

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