SAGE-Hindawi Access to Research
Chromatography Research International
Volume 2011, Article ID 929876, 9 pages
doi:10.4061/2011/929876

Research Article

Stability-Indicating Gradient RP-LC Method for the Determination of Process and Degradation Impurities in Bosentan Monohydrate: An Endothelin Receptor Antagonist

Sanjay A. Jadhav,[1] Shashikant B. Landge,[1] Sonali L. Jadhav,[1] Navanath C. Niphade,[1] Saroj R. Bembalkar,[2] and Vijayavitthal T. Mathad[1]

[1] *Research and Development, Megafine Pharma (P) Ltd., Nashik 422 202, India*
[2] *Department of Chemistry, Deogiri College, Aurangabad 431004, India*

Correspondence should be addressed to Vijayavitthal T. Mathad, drvtmathad@yahoo.co.in

Received 16 June 2011; Revised 29 July 2011; Accepted 2 August 2011

Academic Editor: Cristina Minguillón

Described is a simple, rapid, selective, and stability-indicating RP-LC method for the determination of process and degradation-related impurities of bosentan monohydrate. Chromatographic separation was achieved on Zorbax SB-Phenyl column thermostated at 35°C under gradient elution by a binary mixture of solvent A (60% phosphate buffer, pH 2.5, and 40% methanol) and solvent B (acetonitrile) at a flow rate of 1.0 mL/min. Forced degradation was carried out under acidic, alkaline, oxidative, photolytic, and thermal conditions. Significant degradation is observed in acid and alkali stress conditions. Process- and degradation-related impurities were characterized by mass spectrometry, ^{1}H NMR, and FT-IR spectral data. Validation of the developed method was carried out as per ICH requirements. Regression analysis shows an "*r*" value (correlation coefficient) of greater than 0.999 for bosentan and five potential impurities. This method was capable to detect the five impurities at 0.01% of the test concentration of 1.0 mg mL^{-1}.

1. Introduction

Bosentan monohydrate (4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl) pyrimidin-4-yl]benzene-1-sulfonamide monohydrate), a dual endothelin receptor antagonist (ERA) has molecular formula of $C_{27}H_{29}N_5O_6\ S\cdot H_2O$ with relative molecular mass of 569.64. It is the first orally active drug approved by United States Food and Drug Administrative as Tracleer (65 mg and 125 mg) for the successful treatment of pulmonary arterial hypertension (PAH). Tracleer improves the exercise ability and decreases the rate of clinical worsening in patients with WHO Class III or IV symptoms of PAH, by blocking the binding of endothelin to its receptors, thereby negating endothelin's deleterious effects [1–8]. Further Tracleer has been demonstrated to be effective in remodeling the pulmonary vascular tree through several mechanisms including vasodilatation, antifibrotic and antithrombotic actions [9]. An extensive literature survey revealed that there are several bioanalytical HPLC methods for the determination of bosentan monohydrate and its metabolite in blood plasma, whereas, there are few other literatures disclosed only for the quantitative determination of bosentan in biological and formulation samples [10–13]. The reported HPLC method [13] was not capable to separate the peaks of impurities and bosentan. The literature survey also revealed that there was no stability-indicating RP-LC method for the determination of process and degradation-related impurities formed under the stress conditions in bosentan monohydrate.

In this paper we describe development and validation of related substances method for accurate quantification of five potential process impurities in bosentan monohydrate samples as per International Conference on Harmonization (ICH) recommendations. Intensive stress studies are carried out on bosentan monohydrate; accordingly a stability-indicating method is developed, which could separate various degradation products. The present active pharmaceutical

ingredient (API) stability test guideline Q1A (R2) issued by ICH suggests that stress studies should be carried out on active pharmaceutical ingredient (API) to establish its inherent stability characteristics, leading to separation of degradation products and hence supporting the suitability of the proposed analytical procedures. It also recommends that the analytical test procedures for stability samples should be stability indicating and should be fully validated. Accordingly, the aim of present study is to establish degradation pathway of bosentan monohydrate through stress studies under a variety of ICH recommended test conditions.

Development of an accurate and efficient analytical method for determining the quality and evaluating the impurity profile of drug substances is some of the critical activities carried out during process research and development in order to meet the requirements of various regulatory authorities [14, 15]. Hence, this paper provides a simple, rapid, selective, and stability-indicating method for determining the process and degradation-related impurities in samples of the bosentan monohydrate bulk drug along with its validation as per USP and ICH guidelines [16, 17].

2. Experimental

2.1. Materials and Reagents. HPLC grade acetonitrile and methanol were procured from Merck (India). AR grade of triethylamine, orthophosphoric acid, hydrochloric acid, sodium hydroxide, and hydrogen peroxide were procured from Merck, Mumbai (India). HPLC grade water obtained from Millipore system (Millipore Inc., USA) was used for the analysis. The investigated sample of bosentan monohydrate and its potential process and degradation-related impurities (Figure 1) were received from synthetic laboratory of Megafine Pharma (P) Ltd, Nashik, India.

2.2. LC (Analytical) Instrumentation and Operating Conditions. Waters HPLC (Milford, MA, USA) equipped with Alliance 2695 separations module and 2996 photodiode array detector was used. Zorbax SB-Phenyl (250 mm × 4.6 mm, 5 μm) column thermostated at 35°C was used for the separation. Mixture of 40% methanol with 60% of phosphate buffer (aqueous solution of 0.1% triethylamine, adjusted pH 2.5 with orthophosphoric acid) was used as solvent A, and acetonitrile was used as solvent B. The flow rate and injection volume was 1.0 mL min^{-1} and 20 μL, respectively. The analysis was carried out under gradient conditions such as time (min)/A (v/v), B (v/v); $T_{0.01}$/70 : 30, $T_{5.0}$/70 : 30, $T_{25.0}$/40 : 60, $T_{30.0}$/40 : 60, $T_{35.0}$/70 : 30, and $T_{40.0}$/70 : 30. The data acquired at 220 nm for 40 min was processed by using Empower Pro data handling system. Photodiode array detector in scan mode from 200 nm to 400 nm was used for the analysis of forced degradation samples; the peak homogeneity was expressed in terms of peak purity values.

2.3. Preparation of Solutions. A mixture of solvent A and solvent B prepared in the ratio of 50 : 50 (v/v) was used as diluent in the preparation of analytical solutions. Test sample solution concentration of 1000 μg mL^{-1} was prepared for the determination of related substances (process-related impurities). Stock solution of bosentan monohydrate standard of a concentration 1000 μg mL^{-1} was prepared. Working stock solution of 100 μg mL^{-1} was prepared from the said stock solution for related substance determination. Individual stock solutions of each impurity at concentration about 150 μg mL^{-1} (Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E) prepared in diluent was further diluted adequately to study the validation attributes. The specification limits used for validation studies were 0.15% for the related substances viz., Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E. Bosentan monohydrate working reference standard solution (1000 μg mL^{-1}) spiked with all impurities at a specification level (w/w) was used as resolution mixture solution (RMS). The system suitability solution of impurities was prepared at specification level by diluting the above stock solutions.

Sample of formulation drug was prepared by powdering twenty tablets of bosentan, dissolving an equivalent of 25 mg of active ingredient in diluent in a 25-mL volumetric flask and ultra sonicating for about 15 min. The volume was made up to the mark with the diluent, and filtered through Merck Nylon syringe filter having pore size 0.45 μm. The clear liquid was collected and used for the determination of related substances in the pharmaceutical dosage forms. This solution was ten times diluted for the assay determination in pharmaceutical dosage forms.

2.4. Analytical Procedure. 20.0 μL of blank (diluent), RMS, six replicate injections of system suitability solution and test sample solution were separately chromatographed. A resolution of not less than 1.5 between bosentan monohydrate and Imp-B was set as a system suitability requirement in RMS. The relative standard deviation (RSD) of not more than 5.0% for Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E peak areas obtained from six replicate injections of system suitability solution was used to verify the system precision. All the known related substances Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E in test sample were determined against mean area of respective impurities obtained from replicate injections of system suitability solution.

2.5. Procedure for Forced Degradation Study. Forced degradation study was conducted on bulk drug substance in order to prove the stability-indicating property and selectivity of the established method [18]. Forced degradation of bosentan monohydrate was carried out under acid/base hydrolytic, oxidative, thermolytic, and photolytic stress conditions. Solutions of drug substances were prepared in diluent and then treated with concentrated hydrochloric acid (refluxed for 2 h), aqueous 5 M sodium hydroxide (refluxed for 12 h), and aqueous 6% hydrogen peroxide (kept for 24 h at RT). After the degradation, these solutions were diluted with diluent and analyzed by the proposed method. For thermal stress, sample of drug substance was placed in oven with controlled temperature of 60°C for 8 days. For photolytic stress, the sample was exposed to photolytic conditions for 9 days as per ICH guideline. After the exposure to the above

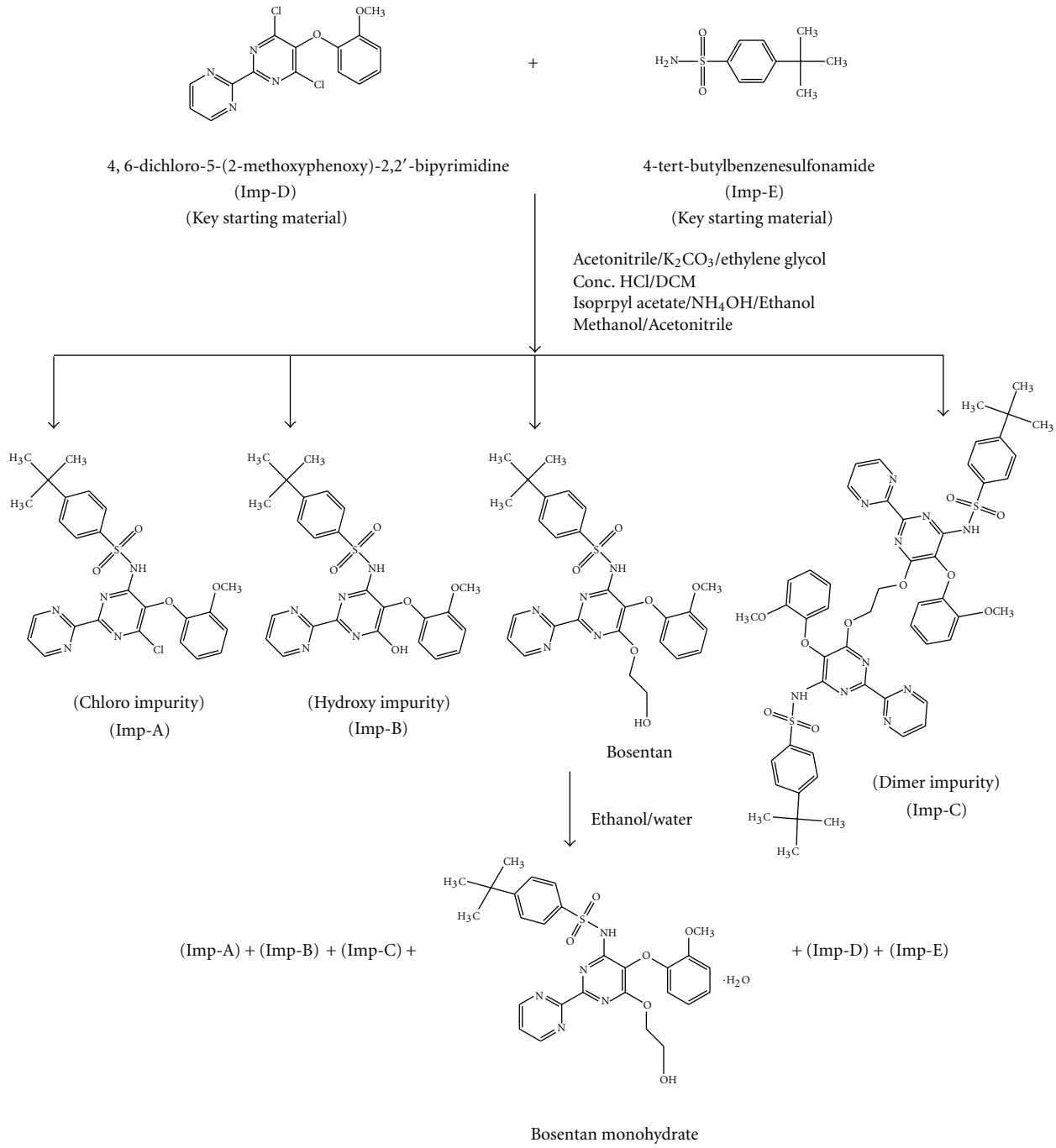


Figure 1: Synthetic scheme of bosentan monohydrate: generation of process-related impurities and degradation-related impurities.

stress conditions, solutions of these samples were prepared by dissolving respective samples in diluent and diluted to the desired concentration and further subjected to analysis using the proposed method. All the stressed samples were quantified for bosentan monohydrate and its impurities. Photodiode array detector was employed to check and ensure the homogeneity and purity of bosentan peak in all the stressed sample solutions. Mass balance of degraded sample was assessed as per Bakshi and Singh. [19] to confirm the amount of impurities detected in stressed samples before and after the application of stress.

2.6. LC-MS Analysis. LC-MS analysis was carried out using triple quadrupole mass spectrometer (API 2000, PE SCIEX) coupled with a Shimadzu HPLC equipped with SPD 10 A VP UV-vis detector and LC AT VP pumps (Foster city, CA, USA). Analyst software was used for data acquisition and data processing. The turbo ion spray voltage was maintained at

Table 1: Mass, FTIR spectral data and 1H NMR chemical shift values.

Name of Impurity	Mass value (m/z) $(M+H)^+$	FT-IR (KBr) absorption bands (cm^{-1})	^1H NMR Chemical shift values, δ in ppm, (multiplicity, integration)
(1) Imp-A	526.01	3379.95, 3053.28, 2960.51, 2866.72, 2903.48, 2960.51, 1248.99, 1347.78, 1176.07, 1663.97, 1080.95, 1551.24, 719.42	8.76-8.77 (d, 2H), 7.45 (t, 1H), 6.86–6.94 (d, 3H), 6.67–6.71 (t, 1H), 6.48–6.50 (t, 1H), 7.48 (s, 1H), 7.09–7.11 (d, 3H), 1.2 (s, 9H), 3.75 (s, 3H)
(2) Imp-B	508.49	3447.92, 3214.78, 3039.63, 2962.43, 2869.34, 2962.43, 1248.00, 1345.77, 1170.86, 1663.72, 1081.03, 1565.46, 759.23	8.99–9.02 (d, 2H), 7.45–7.55 (t, 1H), 11.1 (s, 1H), 6.95–7.03 (d, 1H), 7.07–7.14 (t, 1H), 6.84–6.94 (t, 1H), 9.1 (s, 1H), 8.35–8.45 (d, 2H), 7.40–7.44 (d, 3H), 1.3 (s, 9H), 6.84–6.94 (s, 3H)
(3) Imp-C	1041.20	3063.53, 2963.28, 2837.57, 2869.63, 1574.76, 1558.52, 1335.83, 1257.85, 1257.07, 1163.09, 749.82	8.98 (d, 4H, CH), 8.89 (t, 2H), 4.00 (t, 2H), 3.81 (t, 2H), 6.13-6.99 (m, 8H), 8.29 (s, 2H), 3.72 (s, 6H), 7.25–7.32 (d, 4H), 7.44 (d, 4H), 1.09–1.17 (s, 18H)
(4) Imp-D	350.84	3068.47, 2839.99, 1603.24, 1587.66, 1456.61, 1134.38, 752.09	9.04–9.06 (d, 2H), 7.46–7.49 (t, 1H), 6.78–6.80 (d, 1H), 6.88–6.93 (t, 1H), 7.11–7.16 (t, 1H), 6.99–7.02 (d, 1H), 3.86 (s, 3H)
(4) Imp-E	214.50	1328.75, 1165.96, 1268.00, 1568.44, 832.37, 3268.89, 2963.78, 3363.81	7.50–7.53 (d, 2H), 7.82–7.85 (d, 2H), 1.33 (s, 9H), 4.83 (s, 2H)

S: singlet; m: multiplet; t: triplet; d: doublet.

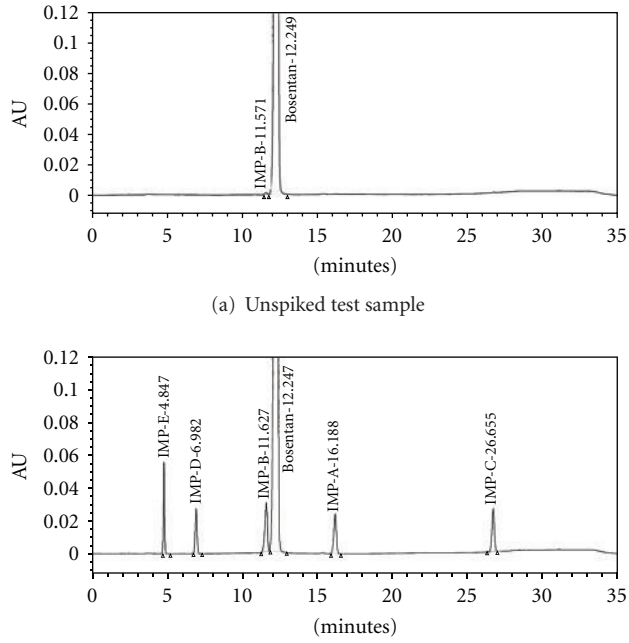
5.5 kV, and temperature was set at 375°C. High pure nitrogen gas was used as auxiliary gas and curtain gas. Zero air was used as nebulizer gas. LC-MS spectra were acquired from m/z 50 to 1200 in 0.1 amu steps with 2.0 s dwell time. Bosentan monohydrate crude sample was subjected to LC-MS analysis. The analysis was carried out on Zorbax SB Phenyl (250 × 4.6 mm, 5 μm) column under gradient elution by a binary mixture of solvent A and B. Solvent A was composed of mixture of ammonium acetate solution at pH 5.4 (0.1% acetic acid, pH adjusted to 5.4 by ammonia), methanol and acetonitrile in the ratio of 45 : 35 : 20 v/v; whereas solvent B was composed of mixture of acetate buffer and acetonitrile in the ratio of 20 : 80 v/v. Detection was carried out at 220 nm, and flow rate was kept at 1.0 mL min^{-1}. Water and acetonitrile mixture in the ratio of 50 : 50 (v/v) was used as diluent. Data acquisition time was 47 min. The gradient program was set as follows: time (min)/A (v/v): B (v/v); $T_{0.0}$/100 : 0, $T_{15.0}$/100 : 0, $T_{25.0}$/45 : 55, $T_{35.0}$/45 : 55, $T_{37.0}$/100 : 0, and $T_{47.0}$/100 : 0.

2.7. Characterization of Impurities

2.7.1. ^1H NMR Spectroscopy. The ^{1}H NMR spectra were recorded on Bruker AV400 (400 MHz) spectrometer using deuteriated chloroform as solvent and tetramethylsilane (TMS) as internal standard.

2.7.2. Mass Spectrometry (MS). Mass spectra were recorded on Waters Micro mass-Quattro micro API mass spectrometer (Waters Corporation, Milford, MA, USA) equipped with a quadrupole mass analyzer. Ions were detected in electron spray ionization with positive ion mode. Spectra were acquired from m/z 60 to 1100 in 0.1 amu steps with 10 numbers of scans.

2.7.3. Fourier Transform Infrared Spectroscopy (FT-IR). FT-IR spectra were recorded for all the five degradation and process-related impurities on Perkin Elmer model-spectrum-100 (California, USA) instrument using KBr pellet method.



(a) Unspiked test sample

(b) Spiked test preparation

Figure 2: Typical RP-LC chromatograms of (a) bosentan monohydrate unspiked test preparation (b) bosentan monohydrate spiked test preparation with known impurities (Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E).

2.8. Synthesis of Bosentan Monohydrate. The reaction scheme for the synthesis of bosentan monohydrate is shown in Figure 1.

3. Results and Discussion

3.1. Detection of Impurities. Laboratory batches of crude bosentan monohydrate were analyzed for their related substances identification using the developed RP-LC method. These samples were subjected to LC-MS analysis. Three related substances were detected in the crude bosentan monohydrate batch sample. The m/z of detected peaks were 508.5[(M+H)+], 527.0[(M+H)+], and 1041.2[(M+H)+]. Based on these observed m/z, starting material and reactants used in the synthetic scheme (Figure 1), the possible structures for related substances were assigned [20]. The impurities (Imp-A, Imp-B, Imp-C Imp-D, and Imp-E) were synthesized and coinjected with bosentan monohydrate to confirm the retention times. All the related substances were well resolved from each other and the representative chromatogram of spiked test preparation is shown in Figure 2. Among the identified impurities, Imp-D and Imp-E are the key starting materials in the process; Imp-B is a process as well as degradation-related impurity; Imp-A and Imp-C are process-related impurities (Figure 1).

3.2. Structural Elucidation. All impurities (Imp-A to Imp-E) were characterized using MS, FT-IR, and NMR spectroscopic techniques. The mass, FT-IR spectral data, and ^{1}H NMR chemical shift values of these impurities are presented in Table 1.

3.3. Development of Chromatographic Conditions. Preliminary experiments for separation of bosentan and all the related substances using RP-LC method under isocratic conditions using Inertsil ODS column (250 × 4.6 mm, 5.0 μm) and employing different eluents were unsuccessful. Further method development trials were performed on gradient mode of separation. The parameters assessed include the type and quantity of organic modifier, the column, the salt concentration, the pH of mobile phase (2.5 to 7), and column temperature. Bosentan monohydrate showed one UV-absorption maxima at about 220 nm, which was selected for further method development. Based on the pK_a value of bosentan (5.46) [21], method development was carried out in acidic mobile phase [22, 23].

The selection of proper stationary phase plays a major role in method development to achieve reproducible results. Initial method development trials conducted using Synergy MAX-RP C12, Hypersil BDS C18, and Cyano columns failed to separate Imp-B from bosentan peak (Figure 3). Further trials were performed using slightly nonpolar column, such as, Zorbax SB (stable bond) phenyl, which was successful as it provides exceptional stability and maximize lifetime and reproducibility under acidic mobile phase conditions. Zorbax stable bond columns are also found compatible with all common mobile phases, including very high aqueous mobile phases [24–26].

The different gradient RP-LC trials were performed on SB-Phenyl column using different buffer (phosphate, acetate and formate) and organic modifier (acetonirile and methanol) to achieve optimum separation. The separation

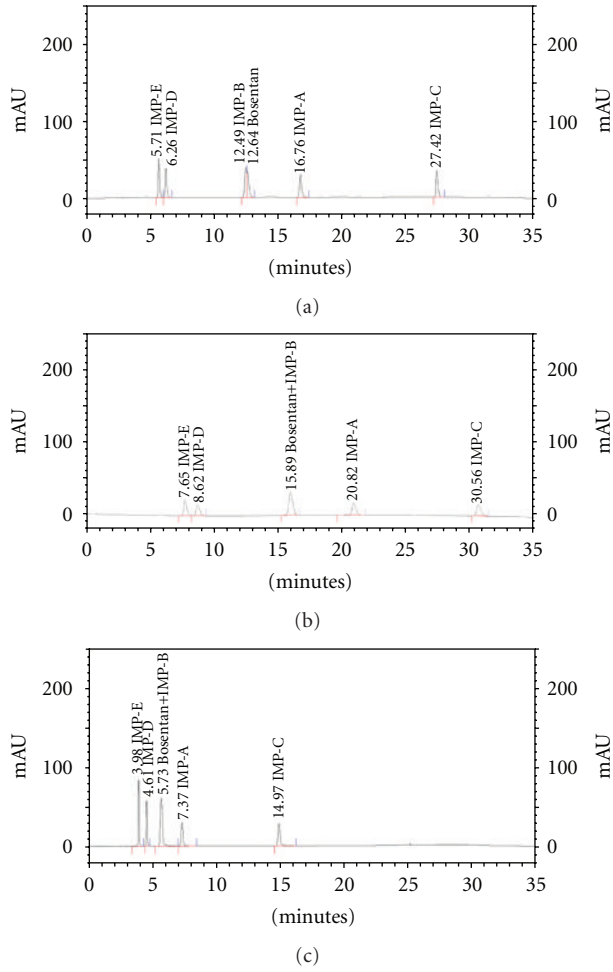
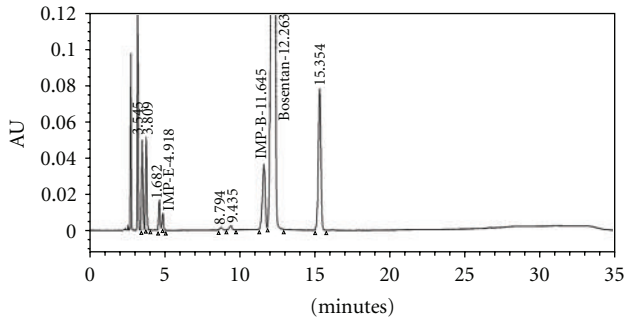


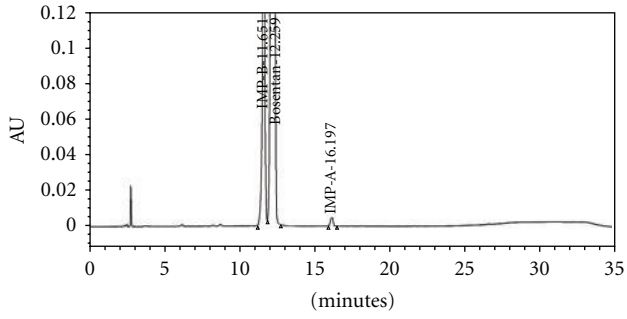
Figure 3: Typical method development RP-LC chromatograms of different stationary phases. (a) Hypersil BDS (250 × 4.6 mm, 5 μ) and (b) synergi Max RP (250 × 4.6 mm, 4 μ), (c) Cyano (250 × 4.6 mm, 5 μ).

between Imp-B and bosentan is achieved only when SB-phenyl column is used, while the separation of Imp A and acid degredants is achieved when buffer pH is maintained at 2.5. Finally satisfactory peak shape and resolution was achieved on Zorbax SB-Phenyl column (250 × 4.6 mm and 5.0 μm particle size) using solvent A (as per discussed in Section 2.2) and solvent B (acetonitrile). The flow rate of the mobile phase was 1.0 mL min^{-1}. The HPLC gradient program was optimized as follows: time (min)/A (v/v): B (v/v); $T_{0.01}$/70 : 30, $T_{5.0}$/70 : 30, $T_{25.0}$/40 : 60, $T_{30.0}$/40 : 60, $T_{35.0}$/70 : 30, and $T_{40.0}$/70 : 30. The column oven temperature was maintained at 35°C with PDA detector set at 220 nm.

It was observed that bosentan, Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E were well separated under the optimized conditions with a resolution greater than 2.0 (Figure 2(b)) and they indicated the specificity of the developed HPLC method for bosentan monohydrate and its impurities (process- and degradation-related impurities) as shown in Figure 4. The optimized method was validated as per ICH guidelines [17, 18]. The developed method was also applied



(a) Acid treated test sample



(b) Alkali treated test sample

Figure 4: Typical RP-LC chromatograms of forced degradation study: (a) acid treated test sample and (b) base treated test sample.

to pharmaceutical formulations and found specific. Thus, the proposed method is simple and could be suitable for the simultaneous analysis of active ingredients in dosage forms.

3.4. Validation

3.4.1. Specificity (Selectivity). During degradation studies, it is observed that the peaks due to degradation products are well separated from the bosentan peak. The peak purity data (purity angle is less than purity threshold) of bosentan monohydrate indicated that it is spectrally pure. Mass balance is a process of adding together the assay value and the levels of degradation products to see how closely these add up to 100% of initial value with due consideration of the margin of analytical error [18]. The mass balance of stressed samples was about 99.0%. Forced degradation study results are given in Table 2.

3.4.2. Linearity. The linearity of peak areas versus different concentrations was evaluated for bosentan and all the related substances using six levels ranging from LOQ to 250% (LOQ, 50, 100, 150, 200, and 250%) with respect to the specification level of impurities. The linear regression data for all the components tested were presented in Table 3. The RF of each impurity was estimated from the slope of the calibration curve for each impurity by using formula: RF of impurity = Slope of the bosentan/Slope of respective impurity.

3.4.3. Limits of Detection and Quantitation (LOD and LOQ). As per ICH Q2R1 guideline, the standard deviation of the peak areas and slope of linearity data limit of detection (LOD) and limit of quantitation (LOQ) of bosentan and related substances were calculated. The LOD and LOQ of bosentan monohydrate were $0.053\,\mu g\,mL^{-1}$ and $0.160\,\mu g\,mL^{-1}$, respectively, whereas the LOD and LOQ for the related substances were in the range of 0.025–$0.068\,\mu g\,mL^{-1}$ and 0.077–$0.206\,\mu g\,mL^{-1}$,respectively. The calculated LOQ concentrations of all the components were further verified for precision by injecting six individual preparations of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, and bosentan monohydrate. The RSD of LOQ precision was in the range of 3.35–8.96%. These limits of quantification levels of the impurities were helpful for the process research work to control the impurities at the accepted level during the optimization of the process. The results are depicted in Table 3.

3.4.4. Precision. System precision for related substances determination was verified by system suitability solution, which was analyzed for six times. RSD of bosentan monohydrate and all impurities peak areas was evaluated and found to be 2.04%. Precision of the method was studied for method precision and intermediate precision. The related substances method precision was demonstrated by analyzing six separate bosentan monohydrate sample solutions that were prepared by spiking the related substances viz., Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E at specification level. The RSD ($n = 6$) for each related substance was evaluated and found to be 0.57–1.22%. In the intermediate precision study, the similar procedure of method precision was carried out by a different analyst, using different instrument on a different day with different lot of same brand column. The percentage relative standard deviation of the results for related substances method was evaluated and found to be 0.59–1.39%. The results of method precision and intermediate precision are compared with each other. The overall RSD ($n = 12$) for percentage of impurities (i.e., Imp-A to Imp-E) was found to be in the range of 0.59–6.49%. The results are reported in Table 3.

3.4.5. Accuracy (Recovery). Accuracy of the method for all the related substances was determined by analyzing bosentan monohydrate sample solutions spiked with all the related substances at four different concentration levels viz., LOQ, 50, 100, and 150% of specification level. Each level has been analyzed in triplicate. Table 3 provides validation data results of related substances of bosentan monohydrate. The recovery of all these related substances was found to be in between the predefined acceptance criteria of 80.0–120.0%.

3.4.6. Stability of Analytical Solution. Sample solutions of bosentan monohydrate spiked with related substances at specified level were prepared and analyzed immediately at different time intervals up to 8 days to determine the stability of sample solution. The sample cooler temperature was maintained at about 25°C and at about refrigerator temperature (8°C). The results from these studies indicated that the sample solution was unstable at room temperature and stable for 8 days at 8°C temperature.

Table 2: Forced degradation study results.

Stress condition	% Assay of bosentan	% of degradant	Observation and mass balance	Peak purity	
				PA	PT
Undegraded	99.95		—	0.180	0.375
Acid hydrolysis (Conc. HCl, 4 h refluxed)	85.84	13.14	Increase in levels of Imp-B (3.28%) and Imp-E (0.41%) and other 9.44% of unknown degradation product formed. (Mass balance: 99.0%)	0.178	0.303
Base hydrolysis (5 M NaOH, 48 h refluxed)	87.32	14.25	Increase in levels of Imp-A (0.47%) and major degradation product i.e. Imp-B (13.78%) formed. (Mass balance: 101.6%)	0.178	0.317
Oxidation (6% H_2O_2 at RT)	98.80	ND	No any known and unknown degradation product formed. (Mass balance: 99.0%)	0.178	0.367
Thermal (60°C, 8-days)	100.31	ND	No any known and unknown degradation product formed. (Mass balance: 100.3%)	0.184	0.377
Photolytic as per ICH	99.16	ND	No any known and unknown degradation product formed. (Mass balance: 99.2%)	0.179	0.368

Mass balance = % assay + % sum of all impurities + % sum of all degradants; PA: purity angle; PT: Purity threshold; ND: not detected.

Table 3: Validation data results of related substances of bosentan monohydrate.

	Related substances results					
Compound	IMP-A	IMP-B	IMP-C	IMP-D	IMP-E	Bosentan monohydrate
Precision; Mean (%RSD)						
Method Precision ($n = 6$)	0.174 (0.57)	0.082 (1.22)	0.169 (1.18)	0.152 (0.66)	0.151 (0.66)	—
Intermediate precision ($n = 6$)	0.170 (0.59)	0.072 (1.39)	0.169 (0.59)	0.154 (1.30)	0.152 (0.66)	—
Overall results ($n = 12$)	0.172 (1.16)	0.077 (6.49)	0.169 (0.59)	0.153 (1.31)	0.151 (0.66)	—
Limit of detection (LOD)						
LOD ($\mu g\,mL^{-1}$)	0.044	0.036	0.025	0.068	0.027	0.053
LOD (% w.r.t. test)	0.004	0.004	0.003	0.007	0.003	0.005
Limit of quantitations (LOQ)						
LOQ ($\mu g\,mL^{-1}$)	0.135	0.109	0.077	0.206	0.081	0.160
LOQ (% w.r.t. test)	0.014	0.011	0.008	0.021	0.008	0.016
Linearity: for related substances LOQ to 250% of specification level						
Correlation coefficient (r)	0.99953	0.99996	0.99975	0.99984	0.99997	0.99998
Slope	28308.09	37412.93	30031.08	24896.49	31706.44	34898.75
Intercept	−1161.25	69.93	−167.92	195.10	172.24	2294.25
Accuracy: mean recovery (%RSD): LOQ to 150% of specification level						
LOQ	93.33 (7.14)	96.67 (5.97)	92.59 (6.93)	106.06 (2.47)	92.59 (6.93)	—
50%	107.92 (1.18)	90.99 (1.71)	98.64 (0.01)	102.22 (3.28)	98.68 (0.00)	—
100%	104.41 (0.74)	93.47 (0.83)	99.55 (1.72)	100.44 (1.01)	100.00 (0.66)	—
150%	102.49 (0.66)	91.62 (1.01)	99.25 (0.95)	99.41 (0.26)	98.54 (0.25)	—

% w.r.t. test LOD LOQ values are in % with respect to test concentration of 1000 $\mu g\,mL^{-1}$.

3.4.7. Mobile Phase Stability. Bosentan monohydrate test sample spiked with related substances at specification level was used to evaluate the mobile phase stability of the method. The bosentan monohydrate test sample was analyzed after 24 hrs and after 48 hrs by using same mobile phase. The content of each impurity was evaluated and compared to the mean results of method precision. The difference between the mean values (after 48 hrs) from the method precision mean results is found to be below 10.0%. The studies indicated that there was no effect on the determination of related substances and the selectivity after 48 hrs. Therefore the mobile phase is stable for 48 hrs.

3.4.8. Robustness. To evaluate the robustness of the developed method, the chromatographic conditions were deliberately altered and the resolution between closely eluting peak

Table 4: Results (%) of formulation tablet analysis and bulk drug batches sample analysis.

Sample source	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	SMUI
Formulation product analysis results						
Formulation-1	0.02	0.11	ND	ND	ND	0.06
Formulation-2	0.03	0.11	ND	ND	ND	0.07
Batch analysis results (%)						
B.NO. BOSPP10001	0.02	0.09	0.02	ND	ND	0.05
B.NO. BOSPP10002	0.01	0.07	0.03	ND	ND	0.04
B.NO. BOSPP10003	ND	0.03	0.02	ND	ND	0.03

ND: Not detected; SMUI: Single maximum unknown impurity.

pair, that is, Imp-B and bosentan monohydrate was evaluated. The flow rate of the mobile phase was 1.0 mL min^{-1}. To study the effect of flow rate on the resolution, the same was altered by 0.1 units, that is, from 0.9 to 1.1 mL min^{-1}. The effect of column temperature on resolution was studied at 32 and 38°C instead of 35°. The effect of buffer pH on resolution was also studied at 2.4 and 2.6 instead of 2.5. All the other mobile phase components were held constant as described above. In all the deliberate varied chromatographic conditions (flow rate, column temperature, and buffer pH), the tailing factor of bosentan monohydrate was less than 1.20, and the resolution between any two peak was greater than 2.0. There was a very minor variation in the resolution and tailing factor results observed in all the robustness conditions illustrating the robustness of the method.

3.5. Application of the Method. The analysis of commercial formulation sample and bulk drug sample indicated that the method is specific and selective for determination of related substances in the formulation and bulk drug samples (Table 4). The developed method is capable for quantitative analysis of bosentan monohydrate in the bulk drug and in a pharmaceutical dosage form.

4. Conclusion

This paper presents a rapid, simple, precise, robust, accurate, and selective gradient RP-LC method that separates the related substances and degradation products of bosentan monohydrate with good resolution. The process- and degradation-related impurities present in bosentan monohydrate sample were identified by LC-MS and characterized by using MS, FT-IR, and ^{1}HNMR spectral data. The developed method was validated to ensure the compliance in accordance with ICH guidelines. Hence, this method can be used for routine testing and stability analysis of bosentan monohydrate drug substance, wherein all the statistical results (percentage, R.S.D., percentage difference, and recovery %) are within the acceptance criteria. The method would not only be useful for routine evaluation of the quality of bosentan monohydrate in bulk drug manufacturing unit but also for detection of impurities in pharmaceutical formulations.

Acknowledgments

The authors wish to thank the management of Megafine group for supporting this work. We would also like to thank colleagues in the division of Research and Development of Megafine Pharma (P) Ltd. for their cooperation in carrying out this work.

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