JOTCS A
Journal of the Turkish Chemical Society
Section: A
Chemistry
TURKISH CHEMICAL SOCIETY

Stability Indicating RP-HPLC Method Development and Validation for Bosentan in Pharmaceutical Formulations

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Abstract: The development and validation of a novel, simple, and quick HPLC technique for measuring bosentan in pharmaceutical formulations was performed. The technique parameters were tuned to be 1 mL/min flow rate, variable column temperature, and a mobile phase combination of methanol-acetonitrile-water (20:50:30 v/v/v) to carry out this study. All measurements were carried out with a UV detector at a wavelength of 272 nm. Specificity, the limit of quantitation (LOQ), limit of detection (LOD), linearity, accuracy, precision, stability, recovery, and ruggedness were all tested. The technique was linear between 0.25 and 20 µg/mL, with precision (RSD%) and accuracy (RE%) of less than 3.0 and 2.7%, respectively. The LOQ and LOD values of method were 0.25 and 0.1 µg/mL, respectively. The 10 µg/mL of standard bosentan solution was found to be moderately stable in acidic and basic settings (0.1 M HCl and 0.1 M NaOH) but unstable in an oxidative environment (H_2O_2 solution; 3%). No interference from tablet excipients was observed in the HPLC method. The approach was successfully applied to pharmaceutical formulations obtained from a local pharmacy store.

Keywords: Bosentan, HPLC analysis, Drug Degradation, Bioanalytical Method Validation

Submitted: October 25, 2021. **Accepted:** March 11, 2022.

Cite this: Turkan D, Yaman M, Atila A, Kadioglu Y. Stability Indicating RP-HPLC Method Development and Validation for Bosentan in Pharmaceutical Formulations. JOTCSA. 2022;9(2):505–12.

DOI: https://doi.org/10.18596/jotcsa.956110.

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INTRODUCTION

Bosentan, also known as (4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidine-2-yl) pyrimidin-4-yl], is a non-selective oral dual endothelin A and B receptors antagonist (Figure 1). Bosentan is an oral medication used to treat pulmonary arterial hypertension (1,2). It has a high protein binding rate (98%) and is quickly absorbed after oral administration, especially to albumin. It has a 45-50 percent bioavailability. Within 3-5 hours, the plasma concentration reaches its maximum (3,4). Hepatic metabolism mostly removes Bosentan, with renal clearance accounting for just 0.9 percent of the total dosage (5).

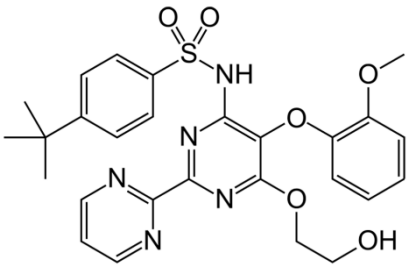

Figure 1: Chemical structure of bosentan.

Various analytical methods have been previously reported for the quantification of bosentan in pharmaceutical preparations including high-performance liquid chromatographic (HPLC) (6-10) and spectrophotometric (11-16) techniques. Besides, four different voltammetric methods for bosentan in pharmaceutical have been reported by

our group (17). The chromatographic separation methods, especially HPLC's, have become prior application in drug stability studies than the other techniques due to requirement of separation of degradation components during the stability tests (18). So far, there is two stability-indicating HPLC method for bosentan is available in the literature (19,20). However, these methods basically focused on the detection of degradation substances, therefore, the total analysis time of the methods are quite long for stability testing of single pharmaceutical substance. Therefore, a rapid stability-indicating HPLC techniques for bosentan are required.

As a result, this study outlines a novel HPLC technique for determining bosentan. This approach aimed to produce an easy and quick assay of bosentan with simple sample preparation and an acceptable analysis time and excellent accuracy. There is no need to remove the medication from the formulation excipient matrix in the suggested technique, which reduces quantization error. After dissolving and filtering, formulation samples can be utilized immediately. The proposed methodologies were utilized to determine the total drug content in bosentan pharmaceutical formulations that are commercially accessible. In addition, the current research covers the invention and validation of a stability-indicating HPLC technique for determining bosentan's stability and quantitative determination in the presence of its degradation products.

EXPERIMENTAL

Reagents and Chemicals

Bosentan was obtained from Actelion Pharmaceuticals (Allschwil, Switzerland). HPLC grade methanol and acetonitrile were obtained from Merck Germany. The mobile phase and solution were made with deionized water that was made fresh every day, filtered (0.45 m), and degassed in the laboratory using a sonicator. In the Turkish pharmaceutical market, bosentan tablet forms were purchased through pharmacies.

Instrumentation and Conditions for Chromatography

The chromatographic apparatus is an Agilent Technologies 1200 series HPLC system with a solvent degassing module (G1322A), quaternary gradient pump (G1312A), autosampler (G1313A), and thermostated column compartment (Agilent, USA) (G1316A).

For chromatographic separation, a reversed-phase ACE C18 column (250 x 4.6 mm, 5µm) was used. The mobile phase was a 20:50:30 (v/v/v) combination of methanol, acetonitrile, and water, and the column was kept at a variable temperature. Isocratic separation was conducted at a flow rate of 1 mL/min and an injection volume of 10 µL. It took 5 minutes to complete the analysis.

Preparation of Quality Control and Standard Solutions

Bosentan was produced as a stock solution in methanol at a concentration of 100 µg/mL. The stock solution was used to provide standard working solutions and quality control solutions (QC). The standard working solutions were prepared in 0.25, 0.75, 2, 5, 10, 15, and 20 µg/mL concentrations. The quality control solutions were made by diluting aliquots of bosentan stock solution to final concentrations of 0.3, 9, and 19 µg/mL.

Pharmaceutical Formulations Procedure

A total of ten bosentan tablets (Tracleer 125 mg and Diamond 125 mg) were precisely weighed and pulverized. A portion of this powder equal to one tablet's bosentan content was weighed and accurately placed into a 100 mL calibrated flask, where it was dissolved in methanol. The flask was then sonicated at room temperature for 10 minutes. In all cases, the resultant solutions were filtered using Whatman 42 filter paper and diluted to achieve a final concentration within the linearity limitations of the proposed technique.

Stress Testing Procedure

Stress testing was performed to assess the molecule's stability and confirm the analytical techniques' stability-indicating power according to ICH standards (ICH Q1A (R2)) (21). Thermal changes with acidic, basic, and oxidizing conditions were taken as the basis to create the experiment.

RESULTS AND DISCUSSION

Chromatographic Optimization

Several mobile phases were tried to determine the HPLC system appropriateness throughout the development and optimization of the technique for determining bosentan in tablets. The use of methanol with various amounts of acetonitrile and water was one of them. A mobile phase of methanol, acetonitrile, and water (20:50:30, v/v/v) was found to provide excellent separation and peak form. Because of the high acetonitrile concentration in the mobile phase combination, the retention period of bosentan decreased, resulting in a quick analysis time. Furthermore, it was discovered that altering the mobile phase's pH value had no significant influence on the intensity analysis time.

The Method's Validation

Specificity, linearity, accuracy, precision, the limit of detection (LOD), the limit of quantification (LOQ), recovery, and ruggedness were all tested in accordance with ICH Q2B guidelines (22).

Specificity

In the assay for bosentan, the effects of common excipients and additives were investigated for probable interference. The most widely utilized excipient in the pharmaceutical business was employed in this formulation. Corn starch, povidon, colloidal silicon dioxide, glycerol dibehenate, magnesium stearate, TiO_2, Fe_2O_3, and talc were shown to have no effect on the analytical results.

Linearity

For bosentan, standard solutions in the range of 0.25-20 µg/mL (0.25, 0.75, 2, 5, 10, 15, and 20 µg/mL) were produced. The calibration curve was created by mapping each sample's peak area against its individual drug concentration (Figure 2). The results were displayed graphically by creating a calibration graph and matching the correlation coefficients on it. All of the calibration curves' correlation coefficients (R) were consistently greater than 0.9997. The least-squares technique was used to compute the linear regression equations using the Microsoft Excel® spreadsheet software, presented in Table 1.

Table 1: Linearity of bosentan.

Range (µg/mL)	LR	R[a]	LOD (µg/mL)	LOQ (µg/mL)
0.25 - 20	0	0	0	0

[a] Based on three calibration curves, LR: Linear regression, R: Coefficient of correlation, y: Peak current, x: Bosentan concentration, LOD: Limit of detection, LOQ: Limit of quantification

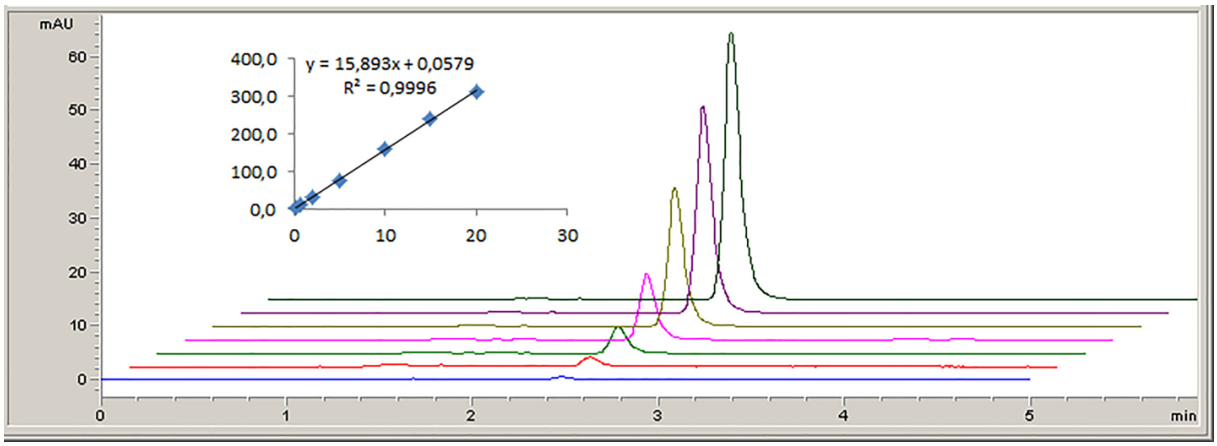

Figure 2: Calibration curve and overlaid chromatorams of bosentan at 272 nm wavelength.

Precision and accuracy

Accuracy was tested and evaluated as relative error (RE%) [(found concentration−spiked concentration)/spiked concentration] × 100%, and the precision of the HPLC method was determined by repeatability (intra-day) and intermediate precision (inter-day). It was expressed as relative standard deviation (RSD%) [(standard deviation /mean concentration) × 100%] of a series of measurements. Six separate tests of quality control solutions at low, medium, and high concentration levels (0.3, 9, and 19 µg/mL) of linearity range were used to evaluate each degree of accuracy and precision (Figure 3). Intra- and inter-day relative standard deviation values were ≤2.60 percent for all concentrations tested, and relative errors were ≤2.67 percent for all bosentan concentrations. Table 2 summarizes the findings.

Table 2: Accuracy and precision results of the proposed method.

Added (µg/mL)	Intra-day			Inter-day		
	Found ± SD	Accuracy (RE%)	Precision (RSD%)	Found ± SD	Accuracy (RE%)	Precision (RSD%)
3	3.11±0.06	-1.00	2.02	3.08±0.08	2.67	2.60
13	13.56±0.32	1.23	2.43	13.27±0.18	2.08	1.36
19	18.62±0.46	-2.00	2.47	19.23±0.12	1.21	0.62
3	3.02±0.07	0.67	1.66	3.07±0.07	2.33	2.28
13	13.25±0.28	1.92	0.38	13.32±0.28	2.46	2.10
19	19.11±0.56	0.58	0.94	19.15±0.56	0.79	2.92

SD: Standard deviation of six replicate determinations, RE%:Relative error, RSD%: Relative standard deviation,

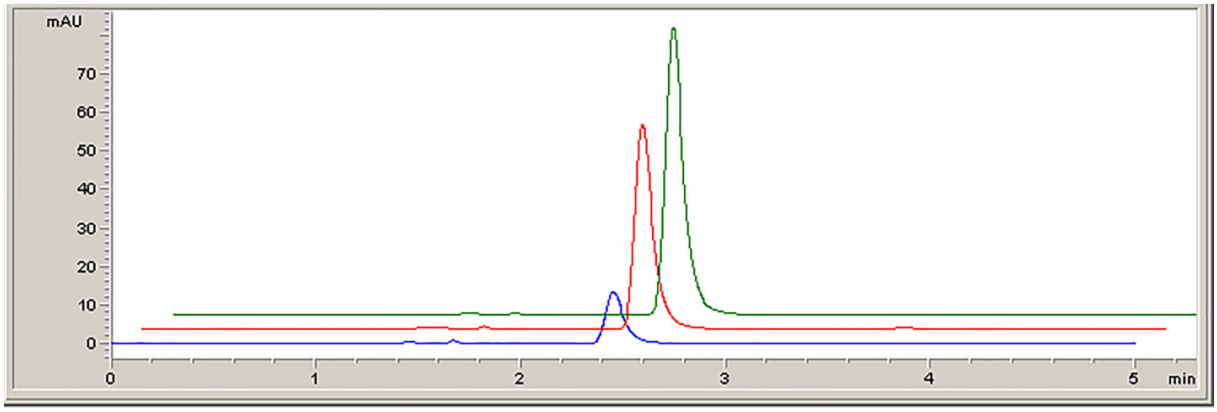

Figure 3: The overlapped chromatograms of quality control solutions of bosentan.

Limits of detection (LOD) and quantification (LOQ)
The LOD and LOQ were established by analyzing the standard solution at successively lower concentrations under the same chromatographic circumstances. The LOD and LOQ were calculated by reducing the signal-to-noise ratios to 3:1 and 10:1, respectively. Table 1 summarizes the LOD and LOQ values of the techniques.

Recovery
Recovery studies were validated using the standard addition method, which involved adding a known quantity (2 µg/mL) of pure pharmaceuticals to pre-analyzed samples that had been spiked with a known amount of standard drug equivalent to 1, 11, and 17 µg/mL. The percent recovery was determined using the formula percent recovery = (T-A)/S100, where T represents the total quantity of drug estimated, a represents the drug provided by tablet powder, and S represents the amount of pure drug added.

The percent recoveries for various spiked levels varied from 99 to 102 percent, with an RSD percent of less than 4%, which is well within the allowed range, confirming the method's accuracy (Table 3).

Table 3: Recovery of bosentan in two tablet formulations.

Drug	Tablet Solution	Added (µg/mL)	Found±SD (µg/mL)	Recovery (%)	RSD %
Tracleer (125 mg)	2 mg/mL	1	3.02 ± 0.12	100.7	3.97
		11	12.68 ± 0.82	99.1	3.26
		17	18.82 ± 0.35	99.1	1.85
Diamond (125 mg)	2 mg/mL	1	3.06 ± 0.06	102.0	1.96
		11	13.21 ± 0.18	101.6	1.36
		17	18.95 ± 0.52	99.7	2.74

Ruggedness
Two separate analysts carried out five sets of experiments for these medicines, and the results in this investigation showed no significant differences. Table 4 shows the results.

Table 4: The results of analyses of bosentan by a different analyst.

Method	Added (µg/mL)	Found (µg/mL) Mean±SD	Recovery %	RSD %
HPLC-UV	3	3.56±0.12	101.7	3.37
	9	9.21±0.14	102.3	1.52
	19	13.32±0.23	102.5	1.73

(n=6) Mean measurements of six replicate determinations.

The Method's Application in Stability Tests

The samples were stable when stored at ambient temperature, +4 °C, and -20 °C refrigeration temperatures for 24 hours (short-term), and refrigerated at +4 and -20 °C for 72 hours, according to stability analyses (long-term). Over the course of 72 hours, there was no substantial change in the analysis. The mean RSD% between peak areas for samples stored under refrigeration (4±1 °C), at room temperature (25±1 °C), and under refrigeration (-20±1 °C) was 1.37%, 1.85%, and 2.05%, respectively, indicating that the drug solution can be stored without degradation over the time interval studied.

Stress testing is also required by the ICH guideline on stability testing of drug substances and products to elucidate the inherent stability characteristics of the active substance and to provide rapid identification of differences that may arise from changes in manufacturing processes or source samples (21). The necessary tests are susceptibilities to acid, alkali, and oxidation hydrolysis stability.

Hydrolysis of acids and alkalis

0.2 mL aliquot of bosentan solution (50 µg/mL) was placed in a tiny rounded flask. 0.8 mL 0.1 M hydrochloric acid or 0.1 M sodium hydroxide was added to the solution. In a boiling water bath, the produced solutions were refluxed for 2 hours. The samples were chilled to room temperature (25±5°C) before being neutralized with an acid or base equal to the amount previously applied. 10 µL of the neutral solution was inserted into the HPLC apparatus (Figure 4).

Oxidation

Into a round-bottomed flask was transferred 0.2 mL of bosentan solution (50 µg/mL). The contents were then combined with 0.8 mL of a 30% hydrogen peroxide solution and allowed to react for 2 hours at room temperature (25±5 °C) with intermittent shaking. In the HPLC system, a volume of 10 µL was injected (Figure 4).

Comparison of the Method

In the literature, there are numerous investigations on the determination of bosentan in pharmaceuticals. From our group, the electrochemical detection methods have been reported for quantification of bosentan in pharmaceutical formulations (17). We were introduced simple, fast and reliable four different voltammetric assay method including cyclic voltammetry (CV), linear sweep voltammetry (LSV), square wave voltammetry (SWV) and differential pulse voltammetry (DPV) for bosentan. The calibration curves were constructed at the range of 5-40 µg/mL for LSV and 5-35 µg/mL for SWV and DPV methods, respectively. Das et al. (13) conducted spectrophotometric research using three distinct techniques for determining bosentan in pharmaceuticals, with a linear range of 0.5-100 µg/mL for all methods. In 0.1 M NaOH, working solutions were produced. Narendra et al. (12) presented a novel spectrophotometric technique for measuring bosentan with a linearity range of 0.1-100 µg/mL and a correlation value of 0.999. Working solutions were produced in the presence of a buffer solution, and pH values were set to 3.5 for measurements. On the other hand, the suggested technique does not necessitate the use of acidic or basic media for the creation of working solutions, nor does it necessitate pH control throughout the analysis. Furthermore, within the prescribed concentration range, measurements were carried out with a higher correlation coefficient. Three distinct spectrophotometric techniques were established to measure bosentan in pharmaceuticals, according to another spectrophotometric approach described by Annapurna et al. (10). For techniques A, B, and C, the linearity ranges were determined to be 1-120, 5-120, and 2-120 µg/mL, respectively. Bosentan was found to be in the range of 10-90 µg/mL by Kumar et al. (11). The linear range was determined to be 0.25-20 µg/mL in our investigation, and it allowed for considerably more exact detection of bosentan. Suganthi et al. (16) presented a technique that combined UV spectrophotometric and HPTLC methodologies. It was said that with the HPTLC technique, very exact findings in a precise linear range could be produced. However, when considering the lengthy preparation procedure of the HPTLC approach, it is possible to conclude that the suggested method is more feasible. Muralidharan et al. (7) proposed a different chromatographic technique and used an LC-UV system, and they were able to do linear readings for bosentan in the concentration range of 5-70 ng/mL. Because of the mistakes that might occur during dilution, this concentration range is excessively sensitive to measuring medication solutions. Furthermore, the method's mobile phase contains an ammonium acetate buffer, and the pH value must be kept constant at 4.5 to correct the measurement of bosentan. In this study, we proposed mobile phase does not contain a buffer solution. With the HPLC-DAD technique, Jadhav et al. (19) conducted stability-indicating research to detect five possible process contaminants in bosentan monohydrate samples and degradation products. Our suggested approach allows for far more exact measuring.

CONCLUSION

For the quantitative measurement of bosentan in pharmaceutical formulations, an HPLC technique was devised and thoroughly validated. The suggested technique allows for the determination of bosentan in pharmaceutical formulations in a quick, easy, accurate, and repeatable manner, with no

interference from excipients. As a result, the devised approach may be utilized for quality control and analysis of bosentan stability samples.

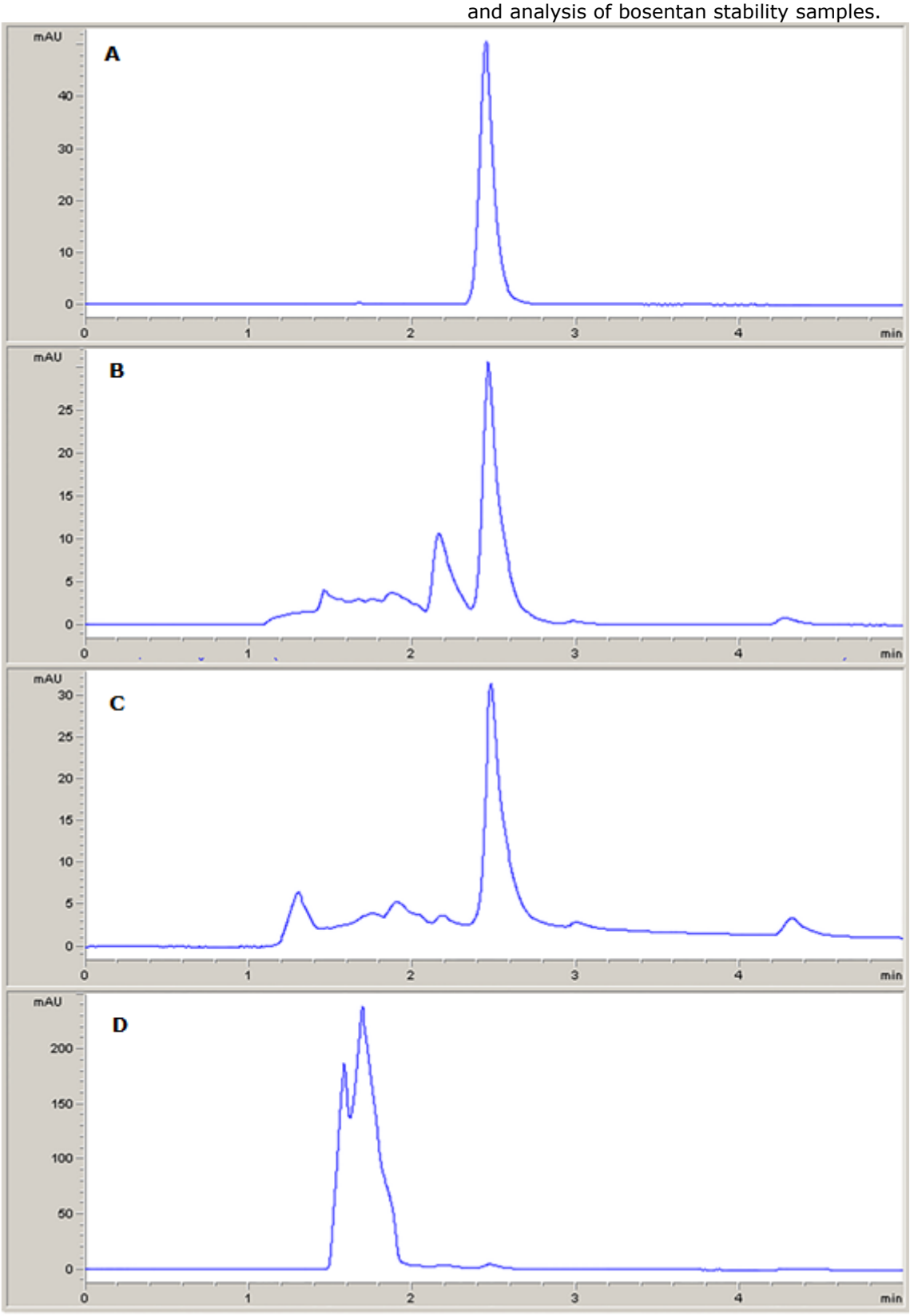

Figure 4: HPLC chromatograms representing degradation behavior of 15 µg/mL bosentan **A)** in no degradation media **B)** in acid **C)** in base **D)** in oxidation.

CONFLICT OF INTEREST

There are no conflicts of interest declared by the authors.

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