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Original Article

Quality By Design Approach for Developing a Validated Liquid Chromatographic Method for Simultaneous Quantification of Bosentan and Sildenafil Citrate with Applications in Dissolution and Robustness Studies

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Abstract

Background: This research aims to establish a validated analytical procedure for the simultaneous quantification of bosentan and sildenafil citrate in a newly encapsulated mini tablet formulation. **Methods**: The chromatography was performed using a reverse-phase column with a mobile phase consisting of acetonitrile and phosphate buffer at pH 4.3 in a ratio of 75:25 (v/v). The flow rate was maintained at 0.9 mL/min, and detection was conducted at a wavelength of 280 nm. **Results**: The developed method demonstrated excellent linearity, with a concentration range of 1–160 µg/mL for both bosentan and sildenafil. Method validation results adhered to acceptable limits established by regulatory guidelines. Additionally, the use of a quality by design approach ensured optimum robustness of the analytical method. **Conclusion**: This validated procedure is suitable for routine quality control of bosentan and sildenafil in the newly developed combined solid oral dosage form, providing a reliable analytical framework for future studies.

Keywords: *Bosentan, Dissolution, Experimental Design, Sildenafil Citrate*

Introduction

Combination medicines targeting various pathways are an attractive approach for treating pulmonary arterial hypertension (PAH) and may offer superior long-term outcomes compared to monotherapy (Lajoie, Bonnet & Provencher, 2017). Bosentan (BSN) was the first non-peptide endothelin receptor (ETA and ETB) antagonist approved for PAH treatment (Masarweh & Bhardwaj, 2024; Nahar *et al*., 2023).P-tert-Butyl-N-[6-(2-hydroxyethoxy)-5-(o-methoxyphenoxy)-2-(2-pyrimidinyl)-4-pyrimidinyl] benzene sulfonamide is its chemical name (Martindale, 2024). With a 50% oral bioavailability and a five-hour plasma elimination half-life, BSN should be given in a controlled-release form to achieve the desired therapeutic effect due to its short half-life (Berger *et al*., 2017; Martindale, 2024). The next drug of our research interest is the orally active, powerful, and selective phosphodiesterase type 5 (PDE5) inhibitor, sildenafil citrate (SDF). The chemical name of the drug is 5-[2-ethoxy-5-(4-methylpiperazin-1-ylsulfonyl)phenyl]1,6-dihydro-1-methyl-3-propylpyrazolo[4,3-d]pyrimidin-7-one citrate (Martindale, 2024; Shih *et al*., 2023). High levels are detected in the lungs. PDE5 blocks cGMP breakdown, relaxes vascular smooth muscle, and increases blood flow (Carbone & Tack, 2018). This enhances the

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vasodilatory effect of nitric oxide in pulmonary hypertension (Bhogal *et al*., 2019). SDF selectively reduces pulmonary artery pressure without causing negative systemic hemodynamic effects (Dural, 2020). Naeije, Richter and Rubin (2021) suggested that PAH is characterized by elevated pulmonary vascular resistance, and early diagnosis with a combination of therapeutic approaches is necessary for improving patient outcomes.

Hence, we attempted to develop a mini tablet dosage form that provides the dual benefit of these drugs in PAH (Cui & Geng, 2021). Sarangi *et al*. (2023) highlighted several advantages of formulating mini tablets, particularly when incorporating one or more active pharmaceutical ingredients. However, it is noteworthy that the quality of such newly developed products must be controlled by employing a reliable analytical technique, such as high-performance liquid chromatography (HPLC) (Ala *et al*., 2023). Chromatographic separation technologies, particularly HPLC, are preferred for medicine stability investigations over other approaches because they are essential for separating degradation products during stability tests (Usta, Timur & Teksin, 2022).

Various HPLC methods have been previously reported in the last several years for the quantification of BSN as well as SDF in pharmaceutical preparations separately (Abdelshakour *et al*., 2021; Anandakumar *et al*., 2018; Gaurkhede and Chandewar, 2017; Panda, Bera & Sahu 2018; Pinto *et al*., 2022; Shahul, Jat & Indulatha, 2017; Sirhan *et al*., 2023; Smerikarova, Bozhanov and Maslarska, 2021; Yaman, Atila & Kadıoğlu, 2022). However, they possess various drawbacks, as depicted in Table 1. Furthermore, no simultaneous HPLC assay methods have been reported for the said drug combination. Hence, the authors are currently making efforts to establish an LC-based method for the newly combined mini-tablet dosage form of BSN and SDF (Sarangi *et al*., 2024). The approach was statistically validated to meet the International Council for Harmonization (International Conference on Harmonisation, 2005) Q2 (R1) guidance and was tested for robustness using an experimental methodology.

Table 1: Comparison of reported and present method for simultaneous analysis of BSN and SDF

References	Method	Remarks	Estimates BSN and SDF simultaneously
Shahul, Jat & Indulatha 2017	HPLC	-Longer run time - Less sensitive linear dynamic range	No
Gaurkhede & Chandewar 2017	HPLC	-Less sensitive linear dynamic range	No
Panda, Bera & Sahu 2018	UFLC	-Less sensitive linear dynamic range	No
Anandakumar *et al*., 2018	HPLC	-Less sensitive linear dynamic range -Higher mobile phase consumption	No
Abdelshakour *et al*., 2021	HPLC	-Demands multiple organic solvents -Uses higher volumes of triethylamine requiring longer stabilization time	No
Smerikarova *et al*., 2021	HPLC	-Higher values of % relative error	No
Yaman, Atila & Kadıoğlu, 2022	HPLC	-Consumption of higher volumes of organic phase; -Co-eluting degradation peaks were observed	No
Pinto *et al*., 2022	HPLC	-Uses multiple organic solvents -Requires longer stabilization time due to use of triethylamine	No
Sirhan *et al*., 2023	HPLC	-Less sensitive linear dynamic range	No
Current	UFLC	-Sensitive linear dynamic range, reasonable and miniaturized use of organic solvents, optimum retention time, avoids use of peak modifiers like triethylamine and eradicates the demand of higher instrument stabilization time, design of experiment based robustness testing	Yes

Method

Care Formulations Limited (Delhi, India) gave a complimentary sample of BSN (quality > 99.14%). Kay Biotech (Delhi, India) gave a complimentary SDF sample (quality > 99.32%). All other chemicals were analytical reagents. S.D. Fine-Chem Ltd., Mumbai, supplied HPLC-grade methanol and potassium dihydrogen phosphate, hydrochloric acid, hydrogen peroxide, orthophosphoric acid, and sodium hydroxide, of AR grade.

Instrumentation

The LC separation was achieved on a binary gradient LC system with Prominence Series Pumps (Shimadzu, Japan) with injection capacity 20 µL and a photodiode array detector. A 250 mm×4.6 mm i.d., 5 µm dimensioned C-18 column was employed as the stationary phase. Thermo-Fisher's TKA-HPLC water system (Germany) produced phosphate buffer water. Design Expert software (Stat-Ease, IN, Minneapolis, USA) was used to study procedure robustness.

Preparation of mobile phase

The authors degassed HPLC grade acetonitrile and phosphate buffer in an ultrasonic water bath for 30 minutes and filtered through a 0.45µ filter.

Preparation of Phosphate buffer

Phosphate buffer was prepared by dissolving 6.8 grams of KH_2PO_4 in a 1000 mL volumetric flask, diluting it with HPLC water to 1000 mL, and adjusting the pH to 4.3 using Orthophosphoric acid.

Preparation of standard and sample solution

BSN and SDF (10 mg each) were dissolved in the 10 mL volumetric flasks using diluent to prepare the standard solutions (each 1000 µg/mL). These stock solutions were then used to create mixed standard solutions and stored at 4°C, 20°C and at room temperature. These solutions were further diluted to 1 mL using a micropipette to obtain concentrations 1, 2.5, 5.0, 10, 20, 40, 80 and 160 µg/mL of both drugs.

Weighing and grinding twenty mini-tablets resulted into a fine powder. A volumetric flask (10 mL) with mobile phase (5 mL) was filled with tablet powder equating to 12.5 mg of BSN and 5 mg of SDF. Prior to the final dilution to a volume of 10 mL using diluent, the contents were subjected to ultrasonication for a duration of 30 minutes. This solution was membrane filtered and stored under 4 °C till further dilution for analysis.

Validation study

Specificity, linearity, accuracy, precision, and robustness were tested to ensure routine applicability. The ICH guidelines were followed in the above studies. Forced degradation studies were performed on both drugs in order to establish method specificity and stability-indicating nature. A suitable aliquot of the analytes was exposed to stress like acid, alkali, peroxide, thermal, and ultraviolet rays. For the acidic and alkaline stress testing, the analytes were exposed to 200 µL of the 0.1M HCl and 0.1M NaOH for 30 min and then neutralised by their equi-volume counterparts. In the case of peroxide stress, 200 µL of 3% H_2O_2 was applied to the analyte solution for 30 min. For thermal and photolytic degradation studies, the analyte solutions were exposed to a thermostatically controlled water bath maintained at 80 °C and UV light at 365 nm inside a UV-chamber, respectively. All these solutions, after a 30 min time period, were made upto to a volume of 1 mL and were analysed by HPLC for a comparison of peak area with the untreated sample solution. The linearity of the procedure was assessed using the averaged (n = 3) peak area of BSN and SDF in mixed solutions. The concentrations of both analytes that were investigated are in the range of 1-160 µg/mL. Plots of the mean areas (y-axis) *vs* the corresponding concentrations (x-axis) were made. Calculations included correlation coefficients with an R^2 somewhat closer to 1. The accuracy of the approach was verified by measuring the recovery of spiked standards from the consistent drug solutions at 80, 100, and 120% of 100% concentration (12.5 µg/mL for BSN and 5 µg/mL for SDF). Hexaplicate determination was used to evaluate repeatability

and intermediate precision for solutions with a concentration of 10 µg/mL of both analytes on multiple days. Six injections of a constant-concentration solution were used to assess procedural accuracy. For both investigations, mean responses, SD, and %RSD were determined to check accuracy.

Chemometrics based robustness testing

A chemometrics-based experimental strategy was utilized, employing a Box-Behnken design (BBD) for the sorted method variables. The Design-Expert program was used to construct a total of 15 tests at three levels for three method variables. The investigation examined the impact of variables on the analyte resolution (Rs) between the two peaks and respective peak areas. The variables Rs and peak areas were designated as the responses to be monitored during the specified experiments. Then, the data were reviewed to improve and create an adequate design space for the approach's reliable performance.

Application to in-vitro dissolution testing and assay

The BSN Sustained Release (SR) and SDF Immediate Release (IR) Mini Tablets (MTs) underwent laboratory testing to assess their dissolution characteristics utilizing USP Type 2 equipment. Following optimization, a solution of 0.1 M hydrochloric acid (HCl) with a pH of 1.8 was chosen for use in the gastrointestinal system. Due to the lack of coating on the tablets, they rapidly decomposed in the gastrointestinal tract. Therefore, a dissolving medium of 0.1 M HCl was used. The effectiveness of dissolution apparatuses relies heavily on hydrodynamics, namely coning, which might impact the results of dissolving studies. To reduce coning, the paddle speed was tuned to 50 revolutions per minute (rpm). A total of 900 mL of media volume was distributed across six baskets, with two additional baskets reserved for replenishment purposes. Prior to processing, the medium underwent degassing by a sonication procedure. At various time intervals (0–150 min), samples were collected at respective time interval. The time intervals for the BSN SR were 1, 3, 6, 9, 12, 15, 18, and 24 h , whereas for the SDF MTs they were 5, 10, 15, 30, 45, and 60 min. The testing samples were acquired by accurately removing 5 mL aliquots from the dissolving jar. In order to sustain the sink condition, the dissolving jar was refilled with 5 mL of fresh media.

The stored sample solutions prepared for the assay were further diluted to obtain a final concentration of 12.5 and 5 µg/mL of BSN and SDF, respectively, and were injected in triplicate onto the column. The average peak areas were determined, and the overall analyte content in the developed dosage form was assessed.

Results

Method development

Initial investigations showed that both BSN and SDF have a higher solubility in acetonitrile (the organic component). The mobile phase pH is essential for separating analytes on the reversed stationary phases. The water-based phase was a phosphate buffer solution containing KH_2PO_4.

An investigation was conducted on a phosphate buffer with a concentration of 0.01 M, examining its behaviour at several pH levels including 3.8, 4.0, and 4.3. The photo diode array (PDA) analysis of BSN reveals distinct absorption peak at a wavelength of 266 nm. In contrast, SDF exhibits a single absorption peak at a wavelength of 294 nm. However, upon comparison (not depicted in the picture), it was discovered that the spectra had a shared absorption at a wavelength of 280 nm.

It was observed that in most instances, the peaks were not ideal or the peaks of the drugs could not be detected, with the exception of pH 4.3, when very symmetrical peaks were achieved. The analyte BSN and SDF eluted at retention times of 4.8 and 2.9 min, respectively (Figure 1a-d). Based on the first experiments, it was determined that a mobile phase of acetonitrile and a 0.01 M KH_2PO_4 buffer with a pH of 4.3, at a flow of 0.9 mL/min and 280 nm detection, is appropriate for conducting more detailed research using chemometrics methods.

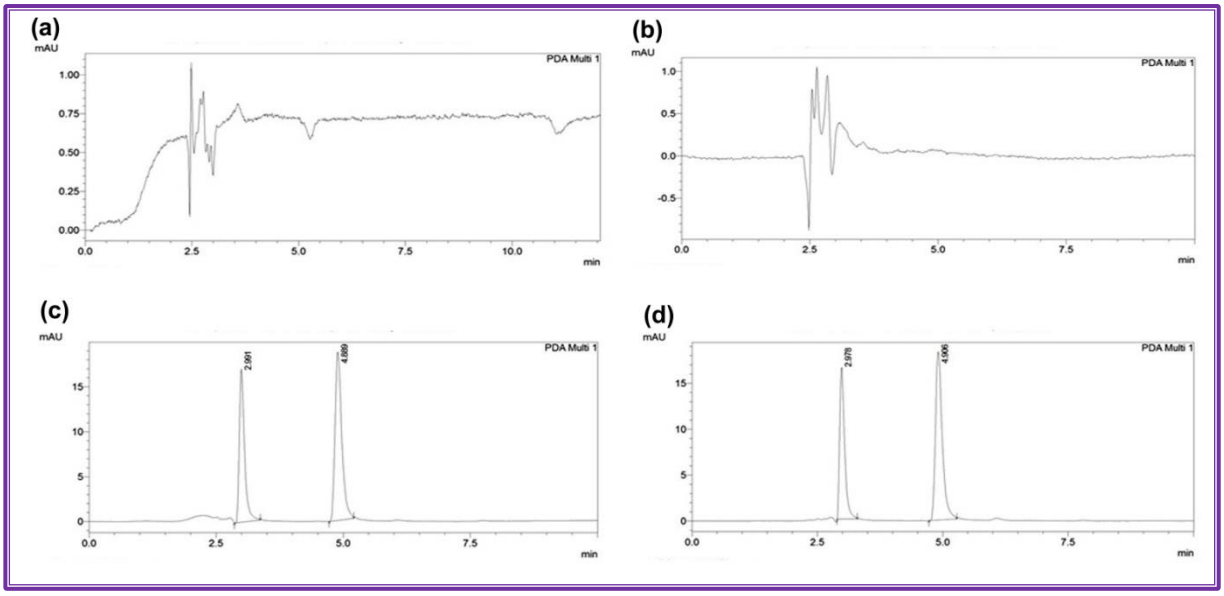

Figure 1: Liquid chromatographic chromatograms obtained for (a) blank, (b) placebo, (c) BSN (Rt = 4.8 min) and SDF (Rt = 2.9 min) in standard drug, and (d)BSN (Rt = 4.9 min) and SDF (Rt = 2.9 min) in developed dosage form

Validation studies

The drugs were stable under acid, alkali, and peroxide conditions and found vulnerable to heat (70 – 70.88%) and photolytic (7.14 – 25.57%) conditions. An extra peak due to possible degradation of analyte and was well resoluted (Rs > 4) from the peak of BSN (Figure 2a-b). A non-interference of degradation peaks with analyte peak ensured procedure specificity. The two drugs were each examined three times across eight concentrations ranging from 1.0 to 160 µg/mL, and the procedure was found linear. The findings in Table 2 depict the precision of the procedure due to the %RSD values being less than 1%. The data suggest that there was no notable reduction in peak area during the storage duration. Table 3 demonstrates that at investigated levels both analytes were recovered well (> 99%), ensuring accurateness of the procedure. The new combination of BSN and SDF was analyzed for its analyte content utilizing the current approach. The data presented in Table 3 demonstrate the selectivity of the chromatographic approach for accurately estimating BSN and SDF in combination dosage form, with an average recovery rate over 97%.

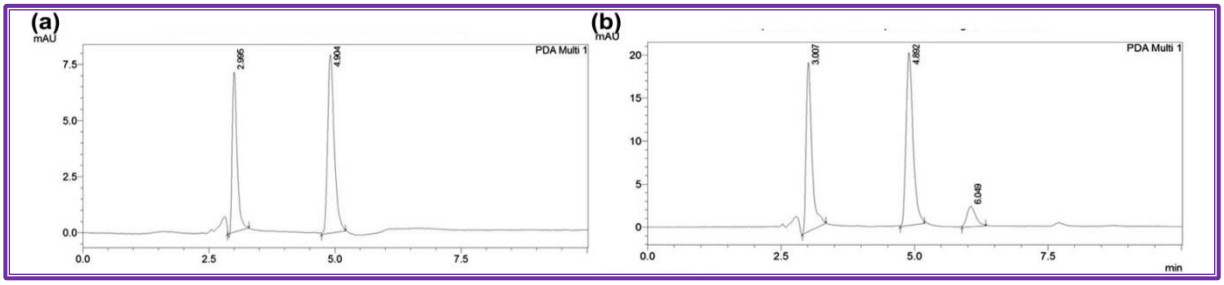

Figure 2: Liquid chromatograms of (a) thermally and (b) photolytically degraded samples of BSN and SDF

Table 2: Method validation results

Parameters	BSN	SDF
Beer's range (µg/mL)	1-160	1-160
Regression equation	27691x-35278	16562x-5353.6
Correlation coefficient (R^2)	0.999	0.999
Accuracy (% recovery[a], %RSD)		
80%	99.9 ± 0.26	101.5,0.7
100%[b]	99.97,0.2	97.93,0.6
120%	100.26,0.3	100.11±0.25
Precision (% RSD[c])		
Repeatability	0.9	0.38
Intermediate	0.67	0.45
Analysis of in-house tablets		
(% Mean[d] ± SD[e])	99.7 ± 0.52	99.89 ± 0.45

[a]percentage recovery stands for mean value obtained from three measurements at each level; [b]100% level stands for the concentration of BSN in the sample solution is 12.5 µg/mL, while the concentration of SDF is 5 µg/mL; [c]%R.S.D. stands for relative standard deviation; [d]Mean stands for mean of three measurements at a concentration of 12.5 µg/mL for BSN and 5 µg/mL for SDF; [e]S.D. stands for standard deviation.

Table 3: Investigated factors and levels of DoE-based robustness test

Run No	Acetonitrile (%)	Flow rate (mL/min)	pH
1	0	-1	1
2	0	1	-1
3	-1	1	0
4	0	1	1
5	-1	0	1
6	-1	-1	0
7	0	0	0
8	1	0	1
9	1	1	0
10	0	-1	-1
11	1	0	-1
12	-1	0	-1
13	0	0	0
14	1	-1	0
15	0	0	0
Coded method factor levels			
Low (-1)	73	0.8	4.1
Mid (0)	75	0.9	4.3
High (+1)	77	1.0	4.5

Designed experiments supported method robustness

First examination of the method variables revealed that three variables required studies in order to create a reliable and precise LC method for BSN and SDF. A total of fifteen experiments were conducted, as shown in Table-3.

The data obtained from these studies were examined using a quadratic polynomial model. During the ANOVA analysis phase, the model's adequateness was assessed in Table 4 and was found suitable for additional studies.

Table 4: Result of ANOVA from the obtained experimental results

Parameter	Resolution (Rs)	Peak Area of BSN	Peak Area of SDF
ANOVA (P = 0.05)	0.0002	<0.0001	<0.0001
F-Value	54.46	85.32	97.36
Adequate Precision	24.92	32.18	31.35

Model appropriateness was confirmed for the objective because of its satisfactory R^2 values, which were greater than 0.9.

The polynomial equations for the responses were formulated according to equations 1 to 3:
Resolution = +8.50 + 0.0737A + 0.1275B - 0.0088C - 1.11AB + 0.1600AC + 0.0075BC - $0.9233A^2$ - $0.7558B^2$ - $0.4833C^2$ …….(1)

Peak Area of Bosentan = +255800 – 3515.63A – 0.8382.13B + 302.25C – 13512.25AB + 3072.00AC – 2884.00BC – $11306.54A^2$ – $20439.54B^2$…….(2)

Peak Area of Sildenafil citrate = +157200 – 6321.38A – 2350.38B + 6626.00C – 13517.50AB – 2969.75AC + 7989.75BC – $9700.08A^2$ – $14867.08B^2$ – $6880.79C^2$ ……(3)

Discussion

The response surfaces presented in Figure 3a-c, which depict the resolution between the drug peaks, revealed that the separation of the drug peaks was stable and well-defined, particularly at the mid-levels of the variables under investigation. This indicates that the method operates optimally under these conditions, ensuring clear differentiation of the analytes. In addition to the resolution data, the response surfaces for the peak areas of Bosentan (BSN) and Sildenafil (SDF), as shown in Figures 3d-f and 3g-i respectively, exhibited tomb-shaped profiles. These response surfaces demonstrated that the peak areas were optimized around the mid-levels of the experimental variables, confirming the robustness of the method at these conditions (Tzimou *et al*., 2024). This level of consistency in the peak areas across the different variable settings underscores the stability and reliability of the method for quantifying both drugs simultaneously (Politis *et al*., 2017). Although two-dimensional contour plots were not included in the graphical representation provided by the authors, the three-dimensional plots viewed from above offer a comprehensive overview of the results (Volta *et al*., 2021). These graphical representations are crucial in validating the accuracy and reliability of the experimental data obtained from the response surfaces (Simeoni *et al*., 2023). The visual correlation between the data and the three-dimensional plots affirms that the method can be relied upon for consistent results under varying conditions (Zagalo *et al*., 2022). By integrating both two-dimensional and three-dimensional plots in the analysis, the study was able to achieve a thorough validation of the experimental data (Jawed & Satish, 2023). The response surface methodology allowed the researchers to assess the impact of the three critical method variables—solvent composition, flow rate, and pH—on the resolution values (Rs) during the simultaneous occurrence of BSN and SDF peaks (Usta, Timur & Teksin, 2022). It was found that these variables exerted a mild to moderate effect on the resolution values, suggesting that while the method is somewhat sensitive to changes in these variables, the separation of the peaks remains reliable within the tested range (Hashmi & Alegete, 2024). This demonstrates that the method has a reasonable degree of flexibility, allowing for slight deviations in experimental conditions without significantly affecting the resolution or accuracy of the drug quantification (Panda & Bera, 2024). Based on the insights gained from these findings, an optimization process was carried out using a combination of both numerical and graphical techniques (Park *et al*., 2022). The goal of this optimization was to identify conditions that would yield desirability values as close to 1 as possible, which is indicative of ideal separation and peak area measurements. The primary aim of this designed experiment was to establish a robust design space that ensures optimal resolution (Rs) between the analytes, while also enabling precise and accurate measurement of peak areas. The overlay plots generated from this analysis showcased the design space region where the method can be expected to perform optimally (Chiarentin *et al*., 2023). This design space is crucial for method robustness, as it identifies the range of experimental conditions under which the method is most reliable and reproducible. From the proposed

optimal solution, it was determined that using 75% acetonitrile as the organic phase, combined with a flow rate of 0.9 mL/min and a buffer pH of 4.3, provides sufficient separation between BSN and SDF peaks. Additionally, this combination of parameters ensures accurate measurement of the peak areas, which is critical for the simultaneous quantification of these drugs in a mini-tablet dosage form. This combination of method conditions was found to deliver the most desirable results, balancing both separation and accuracy effectively.

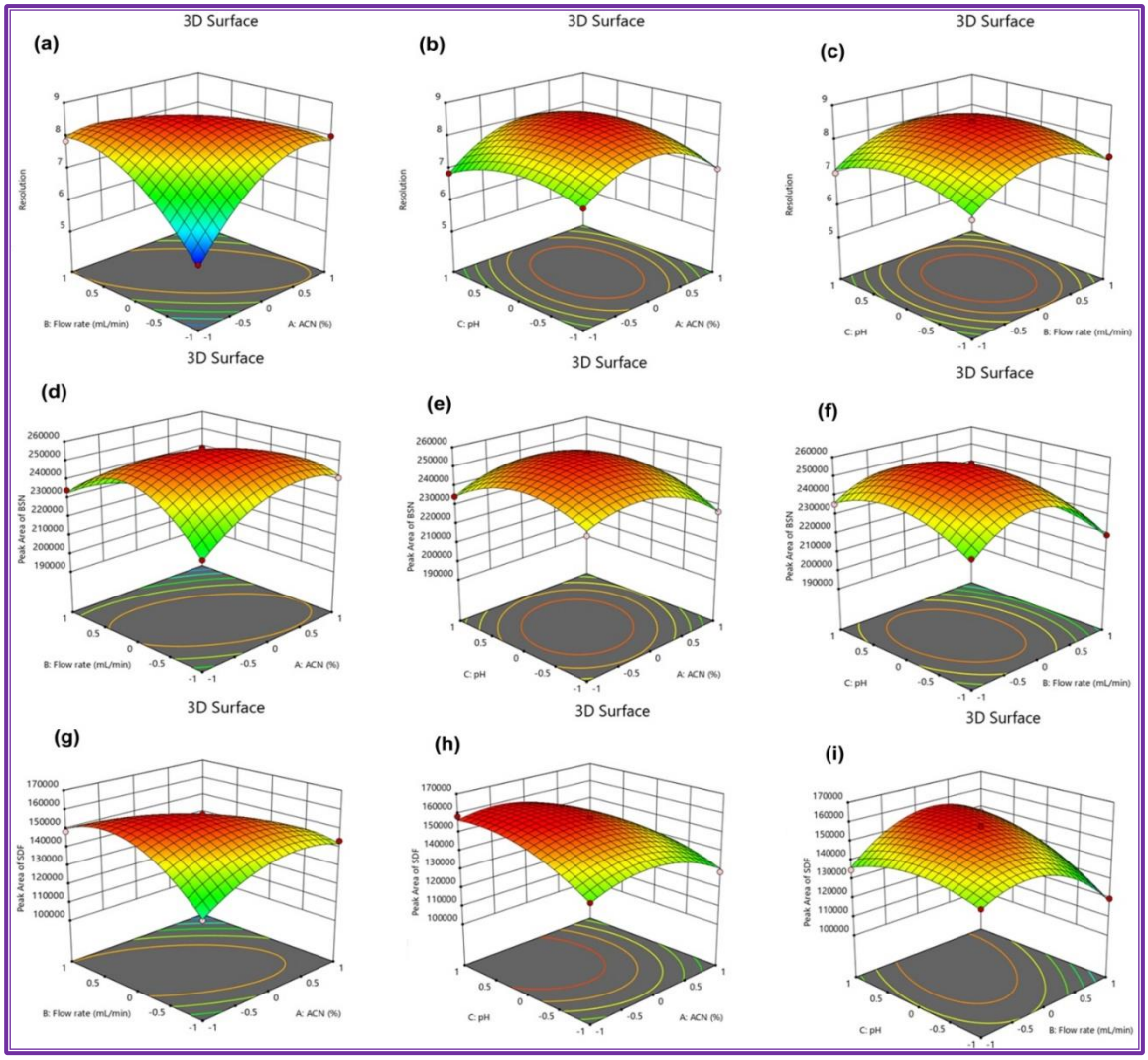

Figure 3: Three-dimensional plots of (a-c) BSN-SDF resolution, (d-f) BSN peak area, and (g-i) SDF peak area

Sarangi *et al*. (2024) carried out the in-vitro dissolution on the encapsulated BSN sustained release mini-tablets (SR MTs) for 24 hours and on SDF immediate release mini-tablets (IR MTs) for 60 minutes. The dissolution profile data, illustrated in Figure 4 a-b showed distinct release patterns for both formulations. The SDF microtubules, which were composed of magnesium aluminum silicate and encapsulated with BSN and hydroxypropyl methylcellulose K15M, exhibited both immediate release and sustained release characteristics (Liu *et al*., 2018; Totea *et al*., 2020). The release of SDF followed an immediate release profile, while BSN exhibited a controlled release pattern, consistent with its formulation design. These findings suggest that the encapsulated mini-tablets can provide both immediate and extended release, making them suitable for PAH treatment, where dual drug delivery over different timeframes is desirable (Özyılmaz & Comoglu 2022). The study successfully developed and validated a robust high-performance liquid chromatography (HPLC) method for the simultaneous quantification of BSN and SDF. The method demonstrated stability, accuracy, and precision, with optimal separation of the drug peaks achieved at the mid-levels of the experimental variables (Swain

et al., 2019). Additionally, the dissolution profiles confirmed that the mini-tablet formulation is capable of delivering both drugs in a manner consistent with their intended release profiles, supporting its potential use in clinical settings for PAH treatment.

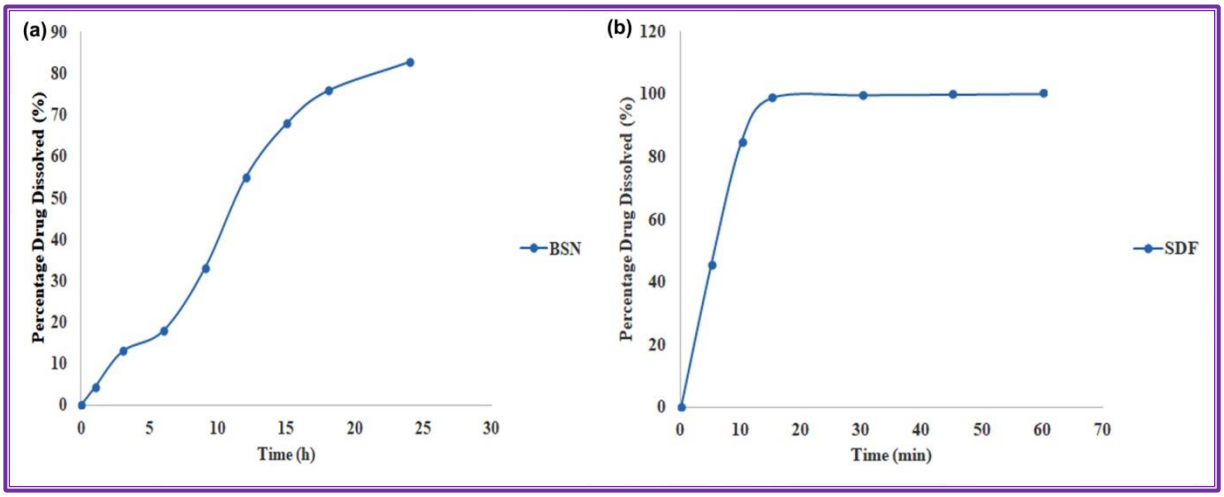

Figure 4: Dissolution release profile of (a) BSN in sustained release and (b) SDF in immediate release mini tablets

Conclusion

In the present study, a novel liquid chromatography method was successfully developed for the simultaneous quantification of bosentan (BSN) and sildenafil (SDF) in a newly co-formulated dosage form. This method was optimized to achieve sufficient resolution between the two analyte peaks and ensure accurate peak area measurements for reliable quantification throughout the product's life cycle. The chromatographic conditions and mobile phase selection were carefully designed to fulfil these objectives. The method was rigorously validated according to ICH Q2 (R1) guidelines, demonstrating adequate linearity, accuracy, and precision for the intended purpose. To enhance robustness, a Box-Behnken Design was employed to study the effects of key variables, including acetonitrile concentration, flow rate, and pH. These factors were found to significantly influence the method's performance, contributing to its overall reliability. In addition, the dissolution profiles of BSN and SDF from the combined dosage form were evaluated, with the results confirming the method's suitability for simultaneous quantification of both drugs. The method's accuracy in quantifying both active pharmaceutical ingredients without compromising on performance underscores its practical application for routine analysis in pharmaceutical quality control. In conclusion, the newly developed and validated analytical procedure offers a reliable and robust tool for the simultaneous quantification of BSN and SDF in co-formulated pharmaceutical products. The method's adaptability, precision, and accuracy make it suitable for routine use in the pharmaceutical industry, particularly for quality control during the production and lifecycle of combination therapies for pulmonary arterial hypertension. This approach can potentially streamline the quality assessment process, reducing the need for separate assays for each drug, while maintaining high standards of regulatory compliance. The method represents a significant advancement in analytical testing for complex pharmaceutical formulations, paving the way for more efficient and effective quality assurance practices.

Conflict of Interest

The authors declare that there is no conflict of interest.

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