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ORIGINAL STUDY

# Greenness Assessment and Development of an Innovative HPLC Method for the Determination of Rifaximin and Sodium Benzoate in Oral Suspension

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## ABSTRACT

The pharmaceutical industry has brought advancement and opportunities. However, it has also presented challenges and complexity that must be addressed. The pharmaceutical industry aims to protect public health by providing economical, effective, safe, and appropriate treatments. The analytical method monitors medication concentrations to ensure efficacy and safety. Chemical preservations protect drugs from microbes until administration. In Refax suspensions, the concentration of rifaximin (Antibacterial, API) and sodium benzoate (chemical preservative) must be determined to ensure the suspension's effectiveness, stability, and safety with regulatory standards.

An innovative HPLC method was created to monitor the concentrations of rifaximin (Antibacterial, API) and sodium benzoate in the Refax Suspension. It was performed by running a mobile phase consisting of a methanol and phosphate buffer on a stationary phase of a C18 column (BDS Hypersil, Thermo Scientific) at a 1 ml $min^{-1}$ flow rate. The detector was adjusted at 230 nm. The optimized method was validated following the ICH guidelines. It was approved to be linear in the range of 25–200 and 2–16 μg $mL^{-1}$, achieving good accuracy results in 99.68% and 99.71% with high sensitivity in terms of LOD concentrations (0.2 and 1.8 μg $mL^{-1}$) for Sodium benzoate and Rifaximin, respectively.

To achieve the objective of conserving public health, the innovative HPLC method's green evaluation combines a variety of tools, including the analytical greenness metric (AGREE), Green Analytical Procedure Index (GAPI), and ECO Scale. The optimized method revealed acceptable results in green assessment.

**Keywords:** Rifaximin, Sodium benzoate, GAPI, AGREE, HPLC

## 1. Introduction

Protecting public health is the overarching objective of the pharmaceutical industry. They aim to do this by ensuring that patients may affordably get successful therapy at the correct dosage. Consequently, two primary concerns in pharmacological treatment are the efficacy and safety of medicines. The drug efficacy depends on the proper dose of the medication that was quantified and monitored by analytical methods. On the other hand, one of the essential aspects of medicine safety is prohibiting microbial contamination of the formula [1]. Chemical Preservatives are the pharmaceutical industry's Secret weapons in Combating rotting. These powerful barriers repel mold, yeast, and bacteria projectiles, guaranteeing medicine preservation until it reaches its moment of administration [2]. Consequently, determining the concentration of rifaximin as API and sodium benzoate as a preservative in Refax®

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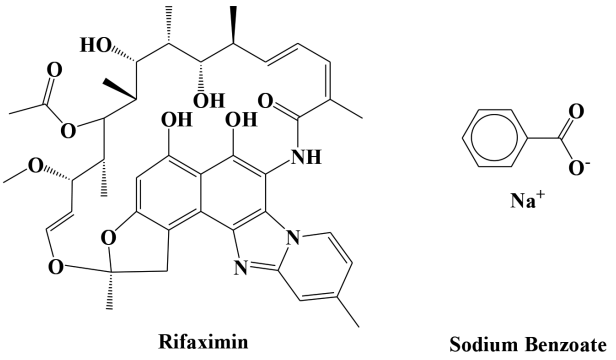



Fig. 1. Chemical structure of RFX and SBZ.

suspension is crucial for insurance. The product is efficacious, stable, and safe within its intended shelf life. It's also paramount for maintaining compliance with regulatory standards and guaranteeing optimal therapeutic outcomes. The analytical methods serve as a critical quality control step in manufacturing, and releasing reflects suspension directly contributes to the project's clinical effectiveness and overall positive patient experience.

Rifaximin (RFX, Fig. 1) is an oral antibacterial derived from rifamycin that treats irritable bowel syndromes, travelers' diarrhea, acute diarrhea, ulcerative colitis, encephalopathy, and Clostridium difficile [3]. The physicochemical properties of RFX reveal that it has pKa values of 4.37 and 4.94 (for strongest acidic) and 11.87 (for strongest Basic), and it is sparingly water-soluble at 0.0074 g/L. The chemical structure of RFX is composed of several functional groups, such as secondary alcohol, secondary amine, aromatic ring, aromatic amine, ketone, and ester [3–5]. RFX acts by lessening the virulence and pathogenicity of bacteria by blocking bacterial translocation across the lining of the gastrointestinal tract. RFX reduced bacterial and cell type-specific adhesion to epithelial cells without altering bacterial count and internalization by downregulating epithelial pro-inflammatory cytokine production. Additionally, RFX influences gut immune signaling [6]. Sodium benzoate (SBZ. Fig. 1) is a salt of benzoic acid commonly used as a chemical preservative. SBZ was discovered in 1875 as a potent antifungal with multiple advantages, such as being cost-effective, easy to make, and not imparting much flavor to beverages, food, or medicines. SBZ is most effective against bacteria, yeast, and fungus. Changes to cell membranes, interference with energy generation, reduction of the intracellular pH, and inhibition of specific enzymes are among the proposed modes of action [7, 8].

Searching the literature, several scientific reports were utilized to quantify RFX in different dosage forms and biological samples using voltammetry [9, 10], UV spectrophotometer [11, 12], Capillary electrophoresis [13], HPTLC [14], HPLC-UV [15–26], LC-MS [27–30], and NMR for elucidation [31]. Other reports described the analysis method of sodium benzoate in pharmaceutical dosage forms and food products using chemometric determination [32], UV spectrophotometer [33], and HPLC [34–40]. After an extensive literature search, no method exists to analyze Rifaximin and sodium benzoate Simultaneously. So, it was aimed to develop and validate an innovative approach to quantify both RFX and SBZ simultaneously.

The scientific community is now interested in green analytical chemistry (GAC). GAC is an area of analytical chemistry that eliminates dangerous compounds and reduces pollution. Reducing energy and waste does not affect analytical performance [41, 42]. This study first substituted safer and more environmentally friendly solvents for mobile phases to give eco-friendly solutions. SBZ and RFX may be determined quickly and efficiently utilizing chromatographic methods without sample extraction, pre-treatment filtering, or derivatization. The environmental friendliness of the chromatographic method was assessed using the GAPI, Green Analytical Procedure Index, and Eco-scale analytical tool [43–45]. Furthermore, laboratory tests that analyze the chemical stability of medicinal products are exposed to degradation that is intentionally caused to evaluate the findings of these tests.

## 2. Experimental

### 2.1. Instruments

HPLC system was utilized; it consisted of a quaternary pump, solvent cabinet, and an auto-injector (Waters Alliance 2695, USA). It is accompanied by a PDA 2996 detector with a flow cell of (1 cm and 1000 psi maximum pressure). The HPLC data was manipulated by Empower 3 software. A BDS Hypersil C8 (150×4.6 mm, 5 μm) or equivalent (Thermo, USA).

### 2.2. Chemicals and reagents

QPS laboratories, kindly provide the working standards for Rifaximin and Sodium benzoate. Methanol HPLC grade, Potassium dihydrogen phosphate, and Heptane-1-sulfonic acid sodium salt were purchased from Fisher Scientific in the UK. Refax® Suspension (B.NO. 232312, Vercure, Egypt) was purchased from the local market. Refax® suspension contains 100 and 8.4 mg per 5 mL of RFX and SBZ, respectively.

### 2.3. Chromatographic conditions

The optimized procedure was carried out using a running mobile phase made up of 60-volume methanol, 40-volume 0.03M potassium dihydrogen phosphate, and 0.014 M heptene-1-sulfonic acid sodium pH to 2.5 adjusted by orthophosphoric acid on a BDS Hypersil C8 (150 × 4.6 mm, 5 μm) or similar (Thermo, USA), at a flow rate of 1 mL $min^{-1}$. Before use, the mobile phase was filtered using a 0.22-micron PTFE filter (Zhejiang Aijiren Technology).

Separation was performed at 40 °C, whereas the PDA was seen at 230 nm in this experiment. The column was conditioned with the mobile phase about 20 minutes before the injection to ensure it would function well at 40 °C. 20 μL of each sample was injected into the chromatography apparatus to load the analytical column.

### 2.4. Preparation of standard stock solutions

The standard stock solution is prepared by combining a solution of RFX and SBZ, each with a concentration of 10000 and 800 μg $mL^{-1}$, respectively. The stock solutions were stored at room temperature. To achieve the desired concentration, accurate portions of stock solutions were transferred into a 10 mL volumetric flask to create the necessary working solutions (25–200% of the nominal concentration (100 and 8 μg $mL^{-1}$) of RFX and SBZ, respectively) that meet the linearity, accuracy, and precision criteria. RFX and SBZ nominal concentrations were 1000 and 80 μg $mL^{-1}$, respectively.

### 2.5. Calibration curve solutions

As a calibration curve, five separate solutions were used to establish linearity. After combining RFX and SBZ standard solutions in 10 mL volumetric flasks, the final concentrations were determined to be 25–200 and 2–16 μg $mL^{-1}$, respectively. This equipment allowed for autosampler injections of each sample onto the BDS Hypersil C8 column at the time. For each concentration being tracked, three chromatographic values were taken. We used the relationship between peak area and concentration to build the calibration curves.

### 2.6. Analysis of pharmaceutical sample solution

Reconstitute a bottle with water as directed in the labeling. Shake well, then pipette 5 mL of the reconstituted, well-dispersed suspension into a 100 mL volumetric flask. Dissolve and complete to volume with 90% Methanol, sonicate for 15 minutes, and filter. Dilute 5 mL of this solution quantitatively with the mobile phase in a 50 mL volumetric flask.

Dilutions were made to attain RFX and SBZ working solutions of 100 and 8 μg $mL^{-1}$, respectively. Chromatography was done as detailed in 2.3. The standard addition method applied the pure standard of each drug to the mixture before completing the chromatographic tests to determine the method's validity.

## 3. Results and discussion

### 3.1. Method development and optimization

An innovative HPLC approach was developed to determine the amounts of RFX and SBX in various laboratory-prepared combinations and pharmaceutical preparations (suspension). The following variables were adjusted to get the best HPLC procedure. After extensively evaluating the drug's range of wavelengths in Fig. 2, scanning was carried out to determine the most beneficial wavelengths for each treatment. It was observed that the best wavelengths for each medication were 230 nm. This wavelength was chosen as the best match for their unique requirements.

Several experiments were conducted using the following mobile phase compositions: a mixture of 0.03 M potassium Dihydrogen Phosphate and acetonitrile in different proportions, ranging from 10 to 40 % Acetonitrile in low (2.2) and high pH (7.4) values. The SBX peak was early eluted, especially in high organic solvent % or High pH value, in contrast to the elution of the RFX peak. Furthermore, there is a variability of retention time and poor symmetry of the RFX Peak in addition to the interference between the SBX peak and placebo peak of citric acid, which is used for pH adjustment, as illustrated in Fig. 3.

To proceed, acetonitrile was replaced with methanol as the organic modifier, and a low pH value of phosphate buffer (2.5) was selected to produce the optimal retention time of SBX and RFX peaks at about 2.6 and 6.3 ± 0.4 min, respectively. Unfortunately, several later trials showed that the retention time of the RFX peak was uncontrolled, so heptane sulphonic acid (HSA) was added in low concentration (0.014 M) to the buffer solution to fix this problem, as shown in Figs. 4 and 5.

Researchers conducted experiments with different flow rates during the previous mobile phases, such as 1 and 1.5 mL $min^{-1}$. The optimal conditions for the separation process were a flow rate of 1.0 mL $min^{-1}$ and a pH of 2.5. These conditions were found to be most suited when employing a mobile phase consisting of 60-volume methanol, 40-volume 0.03M potassium dihydrogen phosphate, and 0.014

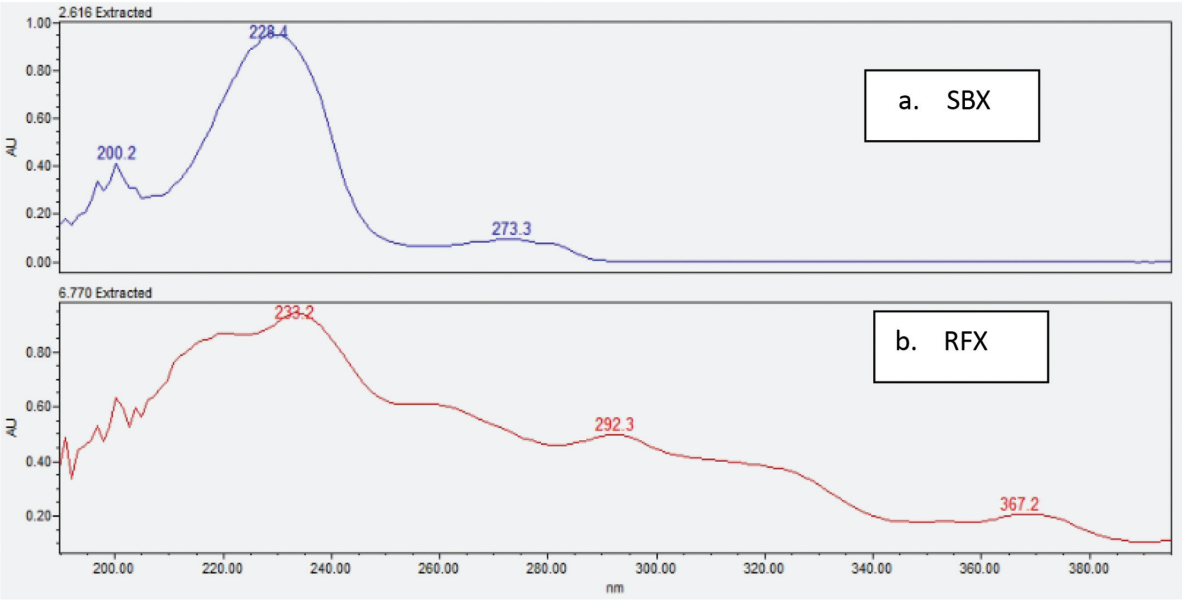



**Fig. 2.** UV Scanning of (a) SBX and (b) RFX in 90% methanol in the range of 400-200 nm.

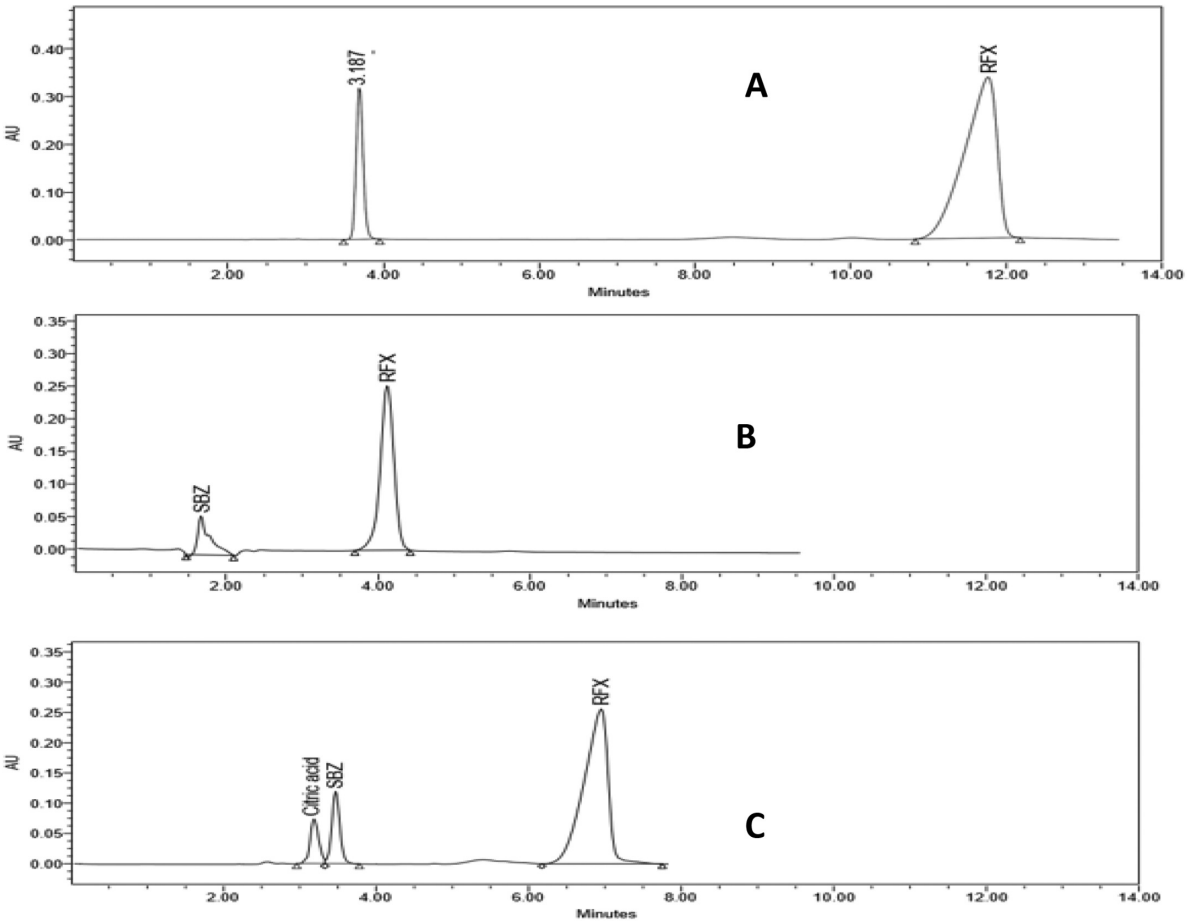



**Fig. 3.** HPLC chromatogram demonstrating the early trial to separate SBX and RFX in Refax Suspension either in Standard or Test solutions (A) Low Acetonitrile% (B) High Acetonitrile% (C) interference between SBZ and citric acid peaks in the test solution.

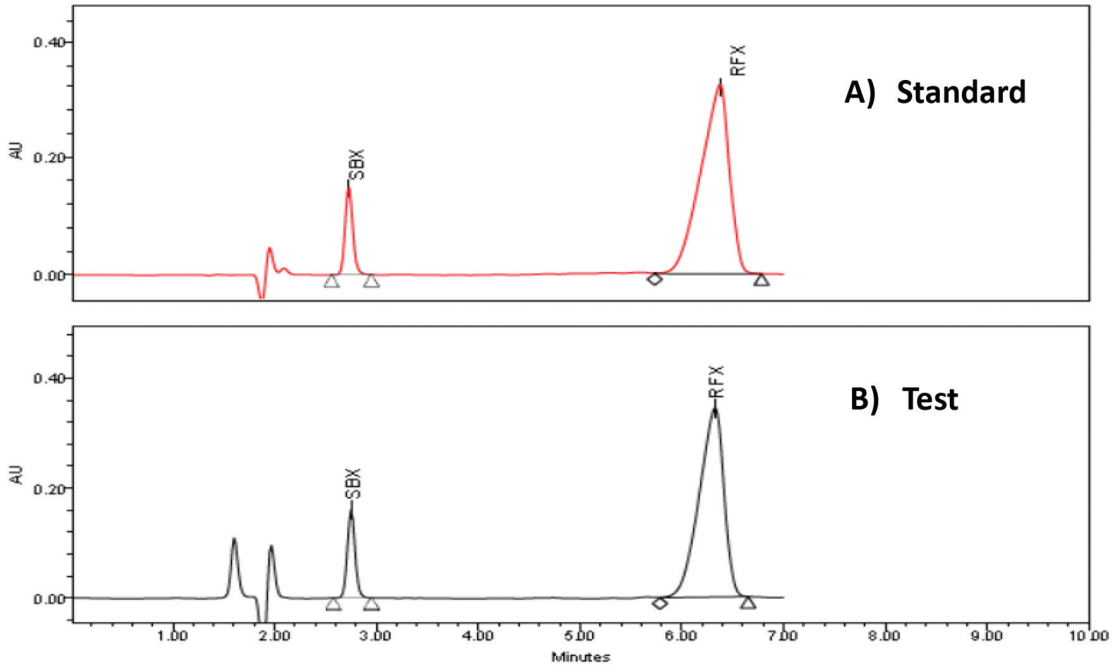



**Fig. 4.** HPLC chromatogram demonstrating the separation of SBX and RFX in Refax Suspension (A) Standard solution and (B) Test solution under the proposed validation Specifications. Mobile phase: Methanol: Phosphate buffer pH 2.5 (60:40) at a flow rate of 1 mL $min^{-1}$.

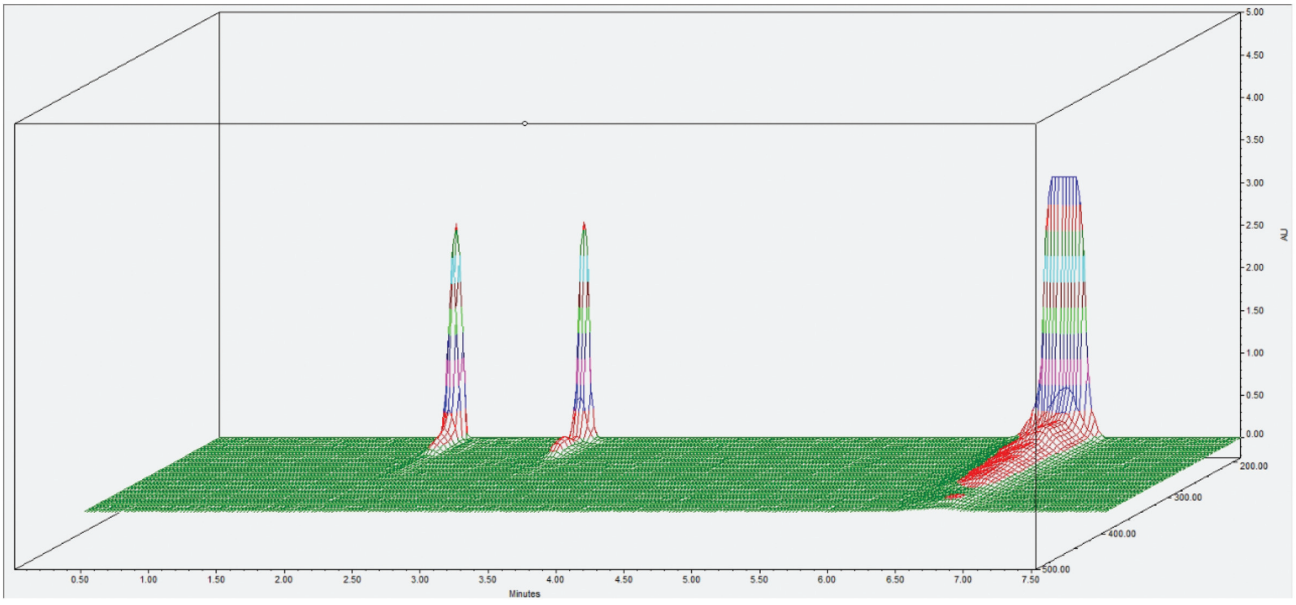



**Fig. 5.** 3D of UV Scanning of (a) SBX and (b) RFX in 90% methanol for test solution in the range of 400-200 nm.

M heptene-1-sulfonic acid sodium pH to 2.5 at a flow rate of 1 mL $min^{-1}$. Separation was performed at 40 °C, whereas the PDA was seen at 230 nm in this experiment. The column was conditioned with the mobile phase about 20 minutes before the injection to ensure it would function well at 40 °C. 20 μL of each sample was injected into the chromatography apparatus to load the analytical column (Figs. 4 and 5).

### 3.2. Method validation

The validity was assessed according to ICH guidelines [47].

#### 3.2.1. System suitability

System suitability parameters include peak symmetry, resolution, theoretical plate count, and system

**Table 1.** System suitability and linearity results for determination of SBZ and RFX using the suggested HPLC approach.

| Parameter | SBZ | RFX |
|---|---|---|
| Retention time (min. ±SD) | 2.60 ± 0.2 | 6.3 ± 0.4 |
| Resolution (Rs) | — | 10.9 |
| Theoretical plates, N* | 5676 | 20888 |
| Capacity factor | 0.74 | 3.07 |
| Symmetry factor | 1.1 | 0.81 |
| Linearity range (μg $mL^{-1}$) | 2.0–16.0 | 25.0–200.0 |
| Linearity equation | y = 87499 × −15047 | y = 60192 × −31014 |
| Correlation coefficient ($R^2$) | 0.9999 | 1.0 |
| LOD (μg $mL^{-1}$) | 0.2 | 1.8 |
| LOQ (μg $mL^{-1}$) | 0.6 | 5.4 |

*N per 10 cm column length.

capacity factor. The system suitability characteristics of the HPLC technique were tested and found to conform to the criteria in the ICH guidelines [47]. The data shown in Table 2 reveals that the resolution is greater than 2, the selectivity is greater than 1, and the number of plates is greater than 2000, as shown in Table 1.

### 3.2.2. Linearity and range

Six different concentrations of RFX (25–200 μg $mL^{-1}$) and SBZ (2–16 μg $mL^{-1}$) were examined for linearity. Calibration curves were generated by comparing the analyte concentrations and peak areas. The graphics in this section (Table 3) demonstrate the derivation of these equations and correlation coefficients ($r^2$). All the method's ($r^2$) values were higher than 0.999, indicating that it is very linear, as shown in Table 1.

### 3.2.3. Limits of detection and quantitation

The limits of detection and quantification (LODs & LOQs) have been determined using the following formulas: $LOD = 3.3 \times SD/S$ and $LOQ = 10 \times SD/S$.

In this formula, (SD) represents the response standard deviation, and (S) is the slope of the calibration curve. The suggested method's strong sensitivity was demonstrated by its low LOD and LOQ values, as indicated in Table 1.

### 3.2.4. Accuracy

The procedures' accuracy was ascertained by measuring various RFX and SBZ concentrations. Table 3 demonstrates the developed approach's accuracy by looking at the mean percentage recoveries (% recovery) and relative standard deviation (% RSD) in Table 2.

### 3.2.5. Precision

Three distinct concentrations of RFX (50, 100, and 150 μg $mL^{-1}$) and SBZ (4, 8, and 12 μg $mL^{-1}$) were analyzed in triplicate on the same day and two following days to determine intraday and interday precision. As demonstrated in Table 4, the developed technique had good accuracy values of % RSD less than 2% for the three concentrations in Table 2.

**Table 2.** Accuracy and precision results for the determination of SBZ and RFX under the proposed chromatographic conditions.

| SBZ* | | RFX* | |
|---|---|---|---|
| | Accuracy | | Accuracy |
| 4 μg $mL^{-1}$ | 99.18 ± 0.20 | 50 μg $mL^{-1}$ | 98.92 ± 0.40 |
| 8 μg $mL^{-1}$ | 99.68 ± 0.14 | 100 μg $mL^{-1}$ | 99.71 ± 0.34 |
| 12 μg $mL^{-1}$ | 99.31 ± 0.35 | 150 μg $mL^{-1}$ | 98.61 ± 0.65 |
| | Repeatability | | Repeatability |
| 4 μg $mL^{-1}$ | 99.5 ± 0.41 | 50 μg $mL^{-1}$ | 100.54 ± 0.20 |
| 8 μg $mL^{-1}$ | 100.52 ± 0.5 | 100 μg $mL^{-1}$ | 99.52 ± 0.5 |
| 12 μg $mL^{-1}$ | 99.51 ± 0.20 | 150 μg $mL^{-1}$ | 99.57 ± 0.40 |
| | Intermediate precision | | Intermediate precision |
| 4 μg $mL^{-1}$ | 100.07 ± 0.22 | 50 μg $mL^{-1}$ | 98.07 ± 0.22 |
| 8 μg $mL^{-1}$ | 100.63 ± 0.10 | 100 μg $mL^{-1}$ | 99.70 ± 0.10 |
| 12 μg $mL^{-1}$ | 10023 ± 0.35 | 150 μg $mL^{-1}$ | 98.33 ± 0.35 |

*Results = Recovery % ± SD "standard deviation".

### 3.2.6. Robustness

The influence of minor adjustments on chromatographic conditions was used to measure robustness. Changes to the mobile phase composition were made, including a 2% change in organic modifier %, a 0.2 change in pH, and a 2 °C change in the temperature. Table 3 shows that the suggested HPLC technique is robust regarding percent recovery and RSD.

### 3.2.7. Specificity

The specificity test may differ depending on the nature of the analytical method and the kind of analyte being evaluated. The specificity was tested by analyzing a blank sample without an analyte, only matrix or solvent, analyzing the tested analytes in the presence of degradation products in different stress conditions, and evaluating the resolution of peaks and recovery results. Selectivity was proven by the high resolution and separation of RFX and SBZ (Fig. 6).

### 3.2.8. Assay of the pharmaceutical dosage form

The newly created method was successfully used to assess the integrated pharmaceutical dosage form (Refax® Suspension) of RFX and SBZ. The technique was also tested using the conventional addition protocol outlined in Table 4. The approach was determined to be precise, and the new standard exhibited high recovery rates.

## 3.3. Greenness evaluation of the suggested method

Investigators calculated penalty points for each analysis step using the analytical eco-scale to test if

**Table 3.** Effect of changing some parameters on resolution and recovery % of SBZ and RFX.

| | Conditions | Changes | SBZ Recovery % | RFX Recovery % | Resolution |
|---|---|---|---|---|---|
| 1 | Proposed method | — | 99.9% | 99.9% | 10.3 |
| 2 | Mobile phase pH | 2.3 | 99.8% | 99.8% | 10.5 |
| 3 | Mobile phase pH | 2.7 | 100.3% | 100.3% | 10.1 |
| 4 | % Methanol | 62% | 100.1% | 100.1% | 9.8 |
| 5 | % Methanol | 58% | 100.3% | 100.3% | 10.5 |
| 6 | Temperature | 38 °C | 99.7% | 99.7% | 10.2 |
| 7 | Temperature | 42 °C | 100.5% | 100.5% | 10.2 |

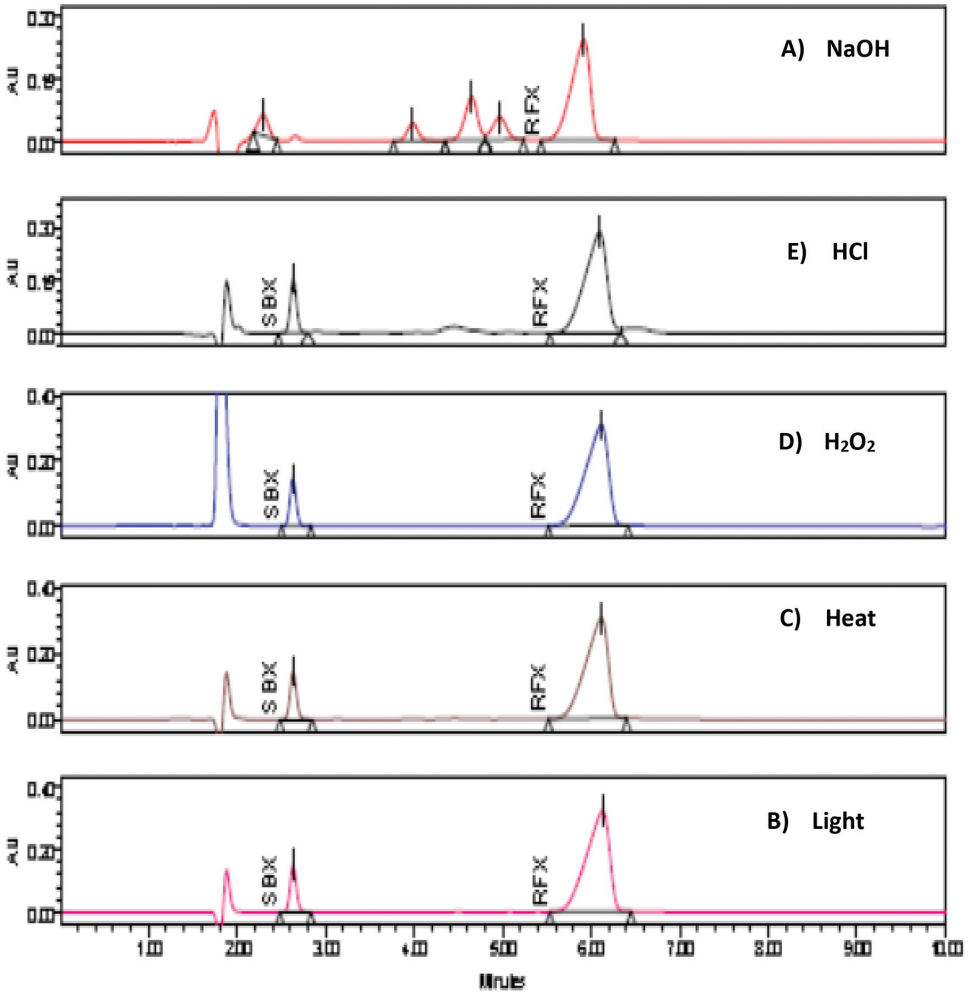



**Fig. 6.** HPLC chromatogram shows SBZ and RFX degradation products under different forced conditions.

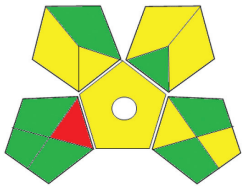
**Table 4.** The results of the application of the proposed method in the determination of SBZ and RFX in their marketed pharmaceutical dosage forms.

| Dosage forms | Drug | Labeled dose per 5 ML | R%* ± SD |
|---|---|---|---|
| Refax® suspension | SBZ | 100.0 mg | 99.10 ± 0.24 |
| | RFX | 8.0 mg | 98.84 ± 0.58 |

*Average percentage recoveries and standard deviations of results (n = 3).

the suggested approach was ecologically beneficial. A grade above 75 signifies a particular green assessment, a degree over 50 indicates good, and a rate of 50 indicates insufficient [45, 49]; see Table 5. A new instrument, the Green Analytical Procedure Index (GAPI), evaluates the environmental friendliness of analytical techniques. GAPI can measure the ecological effect of each analytical process using a five-pentagram symbol. The GAPI pentagram represents

**Table 5.** The penalty points of the proposed method according to the analytical Eco-Scale.

| Reagents/Instruments | ECOSCALE | GAPI | AGREE |
|---|---|---|---|
| Methanol | 8 |  |  |
| Phosphate buffer | 0 | | |
| Heptane sulphonic acid buffer | 0 | | |
| HPLC instrument | 2 | | |
| Occupational hazard | 0 | | |
| Waste | 5 | | |
| Total Penalty points | 15 | | |
| Analytical Eco-Scale Total Score | 85 | | |



low, intermediate, and high impact with green, yellow, and red.

The penalty counts for each reagent were calculated by multiplying the number of Globally Harmonized System (GHS) of Classification and Labeling of Chemicals hazard pictograms by the degree of warning (multiplied by 1) and danger (multiplied by 2). The GHS risk pictograms on reagent bottles make assessing the compounds' risk easier. [43–46, 48, 49]. Table 5 describes the GAPI pentagram interpretation for the proposed chromatographic methodology. The proposed approach was greener than the reported ones. It may be used for routine analysis without harming the environment.

The environmental impact is represented by a color code in the analytical greenness metric (AGREE) that goes from red for a major effect to yellow for a medium effect and green for a minimal impact. A second numerical figure of varying colors is included in the middle of the AGREE graph; this figure provides a general evaluation of the method's "greenness." AGREE considers the procedure's extensive output, the sum of all analytes identified in each run over a given time unit (many hours). Additionally, AGREE assesses the whole environmental performance of analytical techniques using a single numerical number. Finally, while both GAPI and AGREE use color-coded evaluations, GAPI does a better job covering storage, transportation, preservation, and health and safety concerns. AGREE is more concerned with the massive results of analytical methods when evaluating environmental performance, providing only a single numerical number [50].

## 4. Conclusion

Here, an innovative HPLC approach is used to analyze rifaximin and sodium benzoate simultaneously to monitor the drug's concentrations and ensure its safety and efficacy in oral suspension. The optimized method was validated per ICH guidelines and was linear, accurate, precise, robust, and sensitive. It can be applied in quality control routine work in the following tests, including assay of oral suspension, content uniformity, dissolution, and in vitro dissolution. Green assessment of the innovative method was performed using EcoScale, AGREE, and GAPI. The green examination showed promising results and was acceptable as a green method.

## Conflict of interest statement

The authors state that they do not have any conflicts of interest.

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