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Eco-friendly RP-HPLC method development and validation for simultaneous determination of triazole antifungal agent and benzyl alcohol in nanostructured lipid carrier-based bulk and gel formulations

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ABSTRACT

The objective of this study was to create a straightforward, precise, and advanced method for simultaneous measurement of benzyl alcohol (BA) and efinaconazole (EFI) in topical formulations using reverse-phase high-performance liquid chromatography (RP-HPLC) with an Inertsustain C18 column (250 × 4.6 mm, 5 µm). The mobile phase comprised a blend of methanol and buffer-30mM ammonium dihydrogen phosphate (pH 3.00) in a 65:35 ratio, with a flow rate of 1.5 ml/min. The elution was observed at 210 nm. The technique showed linearity within concentration spans of 40–300µg/ml for EFI (R^2 = 0.99992) and 16–120µg/ml for BA (R^2 = 0.99996). The limits of detection for EFI and BA were determined to be 507.49ng/ml and 2838.43ng/mL, respectively, whereas the limits of quantification were 1.54µg/ml for EFI and 8.60µg/ml for BA. The method's validation was conducted following ICH guidelines, achieving accuracy between 98.0% and 102.0%. Both intra-assay and inter-assay precision demonstrated a coefficient of variation under 2.0%. The strength of the created analytical method was further examined by making slight adjustments in wavelength and flow rate. The results from robustness studies indicated that the relative standard deviation and percentage recovery for both BA and EFI fell within the acceptance criteria outlined in the ICH Q2 (R1) guidelines. The established method was exact, dependable, and reliable for concurrent measurement of EFI and BA in pharmaceutical formulations, confirming its suitability for regular analysis.

Keywords: *Benzyl alcohol, efinaconazole, ICH guidelines, PDA-detector, greenness assessment...*

1. INTRODUCTION

Efinaconazole (EFI) is an innovative triazole antifungal compound (chemical name: Based on structure (2R,3R)-2-(2,4-difluorophenyl)-3-(4-methylenepiperidin-1-yl)-1-(1H-1,2,4-triazol-1-yl) butan-2-ol;EFI), with the structure as shown below in **Fig. 1a.** This one works through enforcement of the blockage of the cytochrome P450 enzyme. All these are achieve through the inhibition of ergosterol biosynthesis pathway which aurally affects the fungal cell membranes and its growth [1,2]. In the month of October, 2013, Valeant Pharmaceuticals International got its first non-lacquer topical efinaconazole solution 10% approval outside United States for treating onychomycosis in Canada [35]. Approved by the U.S. Food and Drug Administration (FDA), efinaconazole is intended for topical therapy of onychomycosis [3].

Efinaconazole which is part of triazole drug consists of three nitrogen atoms oriented in a five-membered unsaturated ring. It is highly active against *Trichophyton rubrum* and *Trichophyton mentagrophytes* but in addition to it exhibits good activity against almost all the pathogenic dermatophytes as well as many non-dermatophyte molds [4]. Besides Trichophyton, efinaconazole also targets *Microsporum, Acremonium, Cryptococcus, Epidermophyton, Trichosporon, Fusarium, Pseudallescheria, Paecilomyces, Aspergillus, Scopulariopsis, Candida* species of a small range of triazole agents, efinaconazole, also recognized as KP-103, was identified as having the highest efficacy due to its low affinity to keratin. The parameters of keratin affinity were assessed in a work of Sugiura et al., which compared the antifungal efficacy of efinaconazole with ciclopirox and amorolfine [6].

Benzyl alcohol (BA) is commonly used as an antimicrobial preservative or co-solvent in various pharmaceutical and cosmetic formulations **(Fig.1b)** [7-9]. However, its concentration must remain within specified limits for each type of formulation, as exceeding these limits can lead to severe toxic effects, allergic reactions, and neurological issues [10]. In high concentrations, benzyl alcohol can cause harmful effects such as respiratory failure, vasodilation, hypotension, seizures, and paralysis [11].

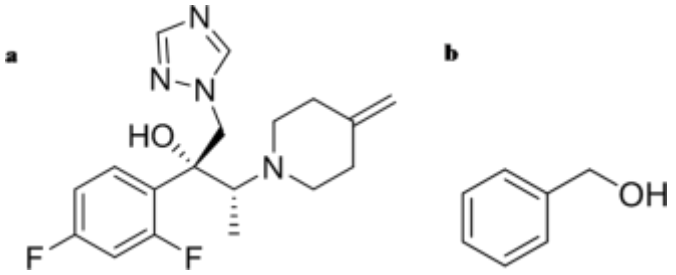


Fig. 1. Chemical structure of (a) efinaconazole (b) benzyl Alcohol.

Various analytical methods, including the HPLC technique individually, have been reported for quantifying efinaconazole [12–15]. LC-MS/MS for quantifying same drug [16–18]. Nonetheless, no simultaneously HPLC method has been documented for its detection. Regarding benzyl alcohol, numerous analytical methods have been developed for its detection in combination with substances such as fulvestrant [19], tolfenamic acid [20], granisetron hydrochloride [21],ethylene glycol monophenyl ether, propyl hydroxybenzoate, butyl hydroxybenzoate, methyl hydroxybenzoate, ethyl hydroxybenzoate [22], benzaldehyde [7], amcinonide [23], benzocaine, and propylparaben [24], along with AG-85 [25]. Furthermore, as the importance of combination dosage forms in current treatments increases due to the development of more combination products, parallel demand for methods for rapid determination of mixtures will increase as well.

This study aimed to address the gap in current analytical techniques by developing a reliable, efficient, and rapidlysimultaneous detection and quantification of efinaconazole and benzyl alcohol in raw materials & formulated productsby reverse phase high performance liquid chromatography (RP-HPLC) method.Based on data or research work, no comprehensive method for the concurrent analysis of these two compounds has been reported in the existing literature. The newly developed RP-HPLC method aligns with the International Council for Harmonisation (ICH) guidelines [26], ensured that it meet global standards for robustness, accuracy, and precision. For the routine analysis, this method was designed to be flexible and reliable for analytical procedure, enabling the simultaneous investigation of both efinaconazole and benzyl alcohol, which is crucial for pharmaceutical products containing these substances.

Using the developed novel RP-HPLC method, the resolution of efinaconazole and benzyl alcohol was achieved under a total run time of 10 min, thus displayed the efficiency of the method. Altogether the research indicated that the used technique has been found effective in determining the concentrations of both species in one and the same drug product easing the problem faced with hitherto approaches whereby individual ingredients are analysed. Due to the ability of the described method to analyse both components concurrently, its benefits over the above methods included a decreased sample amount required for the test, shortened time for processing the sample, and greater dependability of the test outcome. Not only does the use of multiple assays presented its fair share of difficulties and drawbacks, but more importantly they provided numerous assay conditions that could elicit possible errors and variability. Besides, this approach improved the over quality assurance mechanism for developing formulations of pharmaceuticals especially in the expanding market in fixed dose combination and generics. The usefulness of method in enhancing the efficiency and accuracy of the formulations have been helped to drive the need of such formulations while making efficiency enhancement of available resources. The method developed in the present work through RP-HPLC has been found therefore a powerful, accurate and reliable tool for quantification of both efinaconazole and benzyl alcohol simultaneously, which would be very beneficial to the pharmaceutical industry in regulating quality and safety of the combined preparation drugs. It also served as a worthwhile innovation in the areas of pharmaceutical analysis and showed how appropriate resource allocation could help make life saving drugs affordable.

Research and laboratory techniques are shifting towards sustainable and energy-efficient procedures as a result of the chemical community's recognition of the increased significance of green chemistry. This research project works to substitute traditional practices with better environmental alternatives. Green chemistry principles serve as tools to cut down research waste while both lowering solvent use and changing to environmentally friendly chemical systems during research activities. Multiple strategies determine the effectiveness of green approaches against conventional techniques and follow "significance" criteria which meet the twelve principles of green analytical chemistry [41-43].

nalytical chemistry gives particular emphasis to sample preparation because this area most readily accepts green chemistry

principles. The main goal focuses on waste reduction through decreasing the number of procedures needed for sample introduction. Environmental principles from green chemistry help minimize the consumption of energy and reduce the use of chemicals and waste production during necessary sample preparation procedures. The investigation centers its focus on chromatographic techniques since these mixtures separation methods serve as fundamental components for green analytical chemistry [44-47].

The research develops an environmentally beneficial technique to analyze a particular drug substance. This method combines approaches to prevent pollution with sustainable industry practices and pollution control methods as well as environmental safety measures which fulfill environmental and economic objectives and social priorities [48].

The research employs an innovative HPLC method which operates as stability-indicating to conduct the analysis. A greenness assessment of this method utilizes the AGREE standards to evaluate its environmental effects. The assessment takes into account solvent usage, chemical compounds, power requirements and waste outputs as essential evaluation criteria. The method helps environmental preservation by reducing the factors mentioned above.

2. MATERIALS AND METHODS

Chemicals and reagents

Efinaconazole (EFI) sample was received from Chemvon biotechology co. Ltd, China and benzyl alcohol (BA) was purchased from Merck Life Science Pvt. Limited, Mumbai. Ammonium dihydrogen phosphate, triethylamine, ortho phosphoric acid and methanol (LC grade) was purchased from Merck Life Science Pvt. Limited, Mumbai. Highly purified HPLC water was purchased from Rankem, Avantor Performance Materials India Private Limited, Gujarat. Other chemicals and reagents were of analytical reagent grade.

Instrumentation

The LC analysis was used Waters equipment. This included the e2695 Separation Module. It has a quaternary pump to deliver solvents, an autosampler to inject samples, and a variable wavelength programmable PDA detector (2996) to detect across different wavelengths. The system also has a column oven to keep the temperature just right for chromatographic separation, a system controller to manage how the whole system works, and Empower 3,FR3 firmware software to control the system and analyze data. To make sure samples were processed and, they used an automated injector with a 250-µl loop to inject the samples.

Chromatographic Conditions

The mobile phase was composed of a 65:35 v/v combination of methanol and buffer pH-3.00 (30mM ammonium dihydrogen phosphate). 3.45000 g of ammonium dihydrogen phosphate was dissolved in 1000 ml of water; added 1 ml of triethylamine, and the mixture was thoroughly mixed to create the 30mM ammonium dihydrogen phosphate buffer solution, was used diluted orthophosphoric acid to get the solution's pH down to 3.00. The buffer solution was passed through a 0.45µm membrane filters before being sonicated for five minutes to remove any remaining gas. The flow rate of mobile phase was maintained at 1.5 ml per minute to ensure that the progress was at an acceptable rate. The analysis was conducted using Water's e2695 Separation module system, which has a PDA detector set at 210 nm for chromatogram recording. The chromatographic separation was accomplished using a 250 × 4.6 mm, 5µm Inertsustain C18 column, which was maintained at 35°C and sample temperature of 10°C. The injection volume was 20µl.

Preparation of standard solution

Standard stock solutions A

The standard stock solution of EFI was precisely prepared to attain a final concentration of 2000 ppm (2000 µg/ml). Accurately weighed 50mg of efinaconazole and transferred into 25ml of volumetric flak. Consequently, the weighed drug was dissolve in 20mldiluents by using vortexmixer and the make the volume up to the mark with final volume (25ml) with diluent.

Standard stock solutions B

The standard stock solution of benzyl alcohol was precisely prepared to attain a final concentration of800ppm (800µg/ml). 80mg of benzyl alcohol was properly transferred into 100 ml volumetric flask. After that, the weighed amount of benzyl alcohol was dissolved in 70mldiluents by vortex mixer and final volume (100ml) was made up to the mark with diluent.

Furthermore, 5 ml from each of above solutions (A, B) were taken and transferred into 50mlvolumetricflask. 25ml of diluent was mixed and the final volume was made up to the markwith diluent.

Preparation of sample solution

A specific quantity of the gel formulation, equivalent to around 10 mg of the gel formulation was prepared by measuring a

specific quantity of the gel formulation and dissolving in an appropriate solvent. The resulting solution was subsequently diluted to a final volume of 50 ml with a methanol-water mixture (80:20 volume/volume) used as the diluent based on the solubility parameter and mutual miscibility of both polymers. The solution was further filtered through an organism size 0.45µm membrane filter to remove any form of particulate impurity that may have been remaining before it was subjected to the chromatographic procedure. The process was ensured accurate determination of the two active constituents namely efinaconazole and benzyl alcohol in the gel formulation. The filtration step was considered very important for the elimination of possible interference by the contaminants in the successive chromatographic analyses that ensue. All procedures was followed to ensure that no contamination of the sample thereby maintaining stringency of the two elementary entities in the analysis.

Method validation

The HPLC method established for measuring efinaconazole (EFI) and benzyl alcohol (BA) was validated following the ICH Q2 (R2) guidelines. The post-validation process was very stringent to test robust and reliable of the proposed method. Before proceeding with the samples it was necessary to check the suitability of the system for producing accurate analysis through thorough determination of parameters such as system suitability, precision, accuracy, specificity, linearity, limit of quantification (LOQ), limit of detection (LOD), robustness, as well as solution stability. Each parameter was examined to check the corresponding validity of the technique to provide dependable and accurate results. This rigorous validation protocol set in context with ICH guidelines reassured about the robustness, reliability and accuracy of RP-HPLC technique [26, 37]. For this reason, the designed and implemented method was found to be a reliable tool for quantitative estimation of efinaconazole and benzyl alcohol in the gel form. The validation confirmed that the approach is ready for application in other analytical applications in the future.

System suitability

System suitability was estimated using six replicate studies of the drug at concentrations of 80µg/ml for benzyl alcohol (BA) and 200µg/ml for efinaconazole (EFI) to examine the performance of the chromatographic system. The analysis was considered valid if the %CV (percent coefficient of variation) for the peak area and retention times was within ±2%. For subsequent analyses, the standard was ensured that the system was remained stable, accurate and more importantly repeatable in order to provide reliable results. It was revealed that the %CVs falling within the acceptable range settled the fact that the system was observed to be functioned adequately for the required analysis and those variations were made no contribution to the essential chromatographic measurements.The findings showed that the system has been fulfilled the essential criteria for accurate and reliable measurement of both components, confirming the strength of the analytical method [36].

Specificity

The terms specificity refers to the capability of an analytical method to selectively identify an analyte(s) in the occurrence of other compounds in the matrix [27]. In this method, specificity of HPLC has been achieved through ensuring that the analyte peaks have been well separated from any other interfering peaks that may come from the sample matrix or other constituents. To evaluate specificity, 20µl of solutions comprising a blank, placebo, standard, sample, and separate standard for peak identification were injected into the chromatographic system. This has been enhanced the capacity to observe sharp, well-defined peaks for the target analyte eliminating any interferences from other species in the sample matrix on detection of the analyte. The effective separation and recognition of the analyte peaks have been validated the specificity of method, guaranteeing precise quantification free from intervention by other compounds in the sample [37,38].

Stability of analytical solution

The analytical solutions stability was evaluated by examining both the standard and sample preparations at 0 hours and 10°C. A single injection of each solution was performed at different time intervals to track any changes in the solutions over time. The analytical solutions stability was assessed by measuring the peak area and computing the relative standard deviation (RSD) for the peak area from multiple injections. Stable solutions throughout the analysis period were indicated by consistent peak areas with a low RSD value. Possible instability of the solutions may be indicated by changes in peak area or an increase in RSD [28]. This method ensured that the analytical solutions did not undergo any changes. It has been provided reliable and consistent results all through the analysis confirming the correctness of the solutions for accurate quantification and ensuring the stability of the solutions that doesnot affect the outcome [36, 39].

Linearity (calibration curve)

The determination of linearity of the analytical method was determined by creating a stock solution that included EFI (2000µg/ml) and BA (800µg/ml), made by dissolving 200mg of EFI and 80mg of BA in 100ml of diluent. From this stock solution, various concentrations of EFI (from 40 to 300µg/ml) and BA (from 16 to 120µg/ml) were generated for the creation of calibration graphs. Calibration curves were created by injecting six varying concentrations for each substance: EFI (40 to 300µg/ml) and BA (16 to 120µg/ml). For each dilution, all the dilutions were injected and the peak area was recorded for all

concentration levels. The linearity of the response was examined by employing linear regression via the least-squares approach on the data points obtained from the calibration graphs [29]. A calibration plot was created by drawing the peak area versus analyte concentrations, resulting in an equation for quantifying EFI and BA derived from the peak areas measured throughout the investigation. The calibration plots were employed to estimate the correlation coefficients (r^2), intercept and slope [37,40].

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD is the measure of a sample, at which no analysis is possible and LOQ is the measure of the lowest concentration for which quantitative analysis is feasible. This required employing a calibration curve with analyte concentrations near the corresponding limits. In this study, the third statistical method was employed to determine the LOD and LOQ, which was computed using the equations 3.3σ/s and 10σ/s, respectively. In this instance, s represents the gradient of the corresponding calibration curve (10), while σ denotes the standard deviation of the peak area ratios [26, 38]. These values were obtained from the calibration data, confirming that the sensitivity and precision of the methodwere observed within the necessary thresholds for dependable analysis.

Accuracy

The spiking technique was employed to determine accuracy of this analytical method. Placebo samples were enriched with standard solutions of EFI and BA at concentrations of 80%, 100%, and 120% of the anticipated levels. The proposed method was then used to analyze these spiked samples to assess the degree of agreement between the measured values and true or accepted reference values [30]. The experiment was conducted three times for each spiking level to guarantee the reliability of the outcomes. The recovery was determined by contrasting the observed concentrations of analytes in spiked samples with the anticipated concentrations. The method was deemed accurate if the recovery values were observed within the target range, generally 98-102%, confirming the reliable and precise outcomes for measuring EFI and BA amid a sample matrix [39].

Precision

The precision of analytical method was evaluated at both method precision and intermediate precision levels. To assess repeatability, nine replicate injections of mixed standard solutions were performed, either in triplicate or at three distinct concentration levels. This has been included a minimum of six determinations at 100% of the sample solution concentration [31]. The repeatability was determined by evaluating the consistency of the results from these replicate injections. Intermediate precision was also estimated by conducting the same procedure on different days or under varying conditions to ensure the method's robustness. The precision was considered acceptable if RSD of the results was within predefined limits, confirming that the method provides reliable, consistent findings under different conditions and over time. This ascertains the reliability of implemented method for routine investigations.

Degradation studies

The formulation underwent forced degradation experiments to assess the specificity and stability indicating capability of analytical method. Various degradation conditions were applied to evaluate the formulation's stability under different stress conditions. The formulation was exposed to 5 ml of 1 N hydrochloric acid, 5 ml of 1 N sodium hydroxide, and 5 ml of 3% hydrogen peroxide, followed by heating for one hour at 80°C, to induce acidic, alkaline, and oxidative degradation. Additionally, the formulation was neutralized to pH 7.0. For thermal degradation, the formulation was kept at 80°C for 24 hours in an oven [32, 40]. To evaluate photolytic degradation, the formulation was kept in a petri dish and irradiated to sunlight (direct) for seven days. The percentage assay of both EFI and BA in the untreated and degraded samples was calculated, and the peak purity of each component was investigated to confirm the specificity and stability of the method under these conditions.

Robustness

According to the ICH guidelines, the robustness of an analytical procedure refers to its potential to persist unaffected by deliberate and small changes in parameters [33, 36-37]. The robustness of the analytical method was evaluated to assess the impact of small, intentional variations in chromatographic conditions on the analysis of EFI [33]. Various chromatographic parameters were systematically varied to determine their effect on the method’s performance. These parameters included flow rate (1.35, 1.50, and 1.65 ml/min), mobile phase ratio (25:75, 35:65, and 45:55), detection wavelength for EFI and BA (207, 210, and 213 nm), column oven temperature (30°C, 35°C, and 40°C), and pH (2.80, 3.00, and 3.20). Each of these variations was tested to observe any changes in peak resolution, retention time, and peak area. The robustness was considered acceptable if the method provided consistent and reliable results across the different conditions. This evaluation also guaranteed that the method was still valid even if small changes to the chromatographic parameters occurred, which would validate it for routine use in analysis.

3. RESULTS AND DISCUSSION

Method development

Efinaconazole is hydrophobic (insoluble in water) in nature, but it dissolves in organic solventslike ethanol, acetonitrile and methanol [34]. This research work presented the development & validation of a new RP-HPLC method for the determination of BA and EFI with the intention of ensuring a precise, linear, accurate and robust analysis. The method was modified concerning mobile phase, column type and wavelength to provide impressive and fruitful separation and sensitiveness. Particular concern was paid to the elements such as the peak interference, peak identity, the peak shape regularity or symmetry, as well as the retention time to ensure the perfect separation of the analytes. To make peaks sharper and have more space between them, a maximum level of interference was avoided and to have a single ingredient represented by each peak; peak purity was checked. These optimizations of peak shape were found able to produce narrow, symmetrical peaks thus increasing the sensitivity and accuracy of the method. While developing the method, experiments with the mobile phase comprising of acetonitrile, methanol and a buffer were resulted in an asymmetric peak with USP tailing factor of not more than 2. However, the ratio of USP tailing was kept below 1.5, which also contributed to good peak symmetry and reasonable responses in terms of adherence. The flow rate of 1.0 ml/min has been provided a drug retention time of more than 10 minutes which was deemed to be loss making. Since minimization of mobile phase solvent wasobserved achievable whenever flow rates and the run time was decreased, flow rate of 1.5ml/min was adopted to demonstrate the drug's elution time of 7.1minutes and the benzyl alcohol of 2.7minutes as shown in Fig. 2 below. This has been proved to be faster and cheaper for more regular tests on a large number of samples. The best peak symmetry and shape were achieved with a mobile phase comprised of 65:35 v/v (Methanol: Buffer) makes this method appropriate for use in real samples.

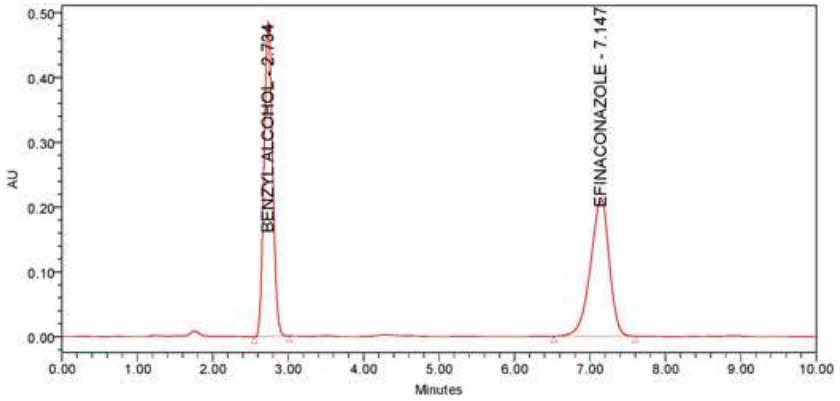


Fig. 2.Symmetry peak of benzyl alcohol and efinaconazole

Method validation

System suitability

Six repeated injections of the standard solution were analyzed using the HPLC instrument to evaluate parameters such as resolution, retention time, column theoretical plate count and repeatability. The standard chromatogram, (as shown in Figure 2), was used for comparison. Statistical analysis revealed that there were no significant variations in key performance metrics, including peak area, retention time, the theoretical plate count (which was>2000 theoretical plates), and the tailing factor (which was < 2.0). Furthermore, the % RSD for these parameters was determined to be under 2.0%, indicating excellent precision and consistency across the injections. The obtained results were confirmed the suitability and reliability of the method for the intended investigation, with minimal variability in the measured values (refer to Table 1 for detailed results). The findings were demonstrated the robustness of the HPLC method for accurate and reproducible measurements.

Table 1. System suitability study of efinaconazole and benzyl alcohol

	Efinaconazole (200 µg/ml)			Benzyl Alcohol (80 µg/ml)		
	Mean (n=6)	SD	%RSD	Mean (n=6)	SD	%RSD
Retention Time (min)	7.130	0.006	0.09	2.716	0.006	0.24
Peak area	3678127.4	16397.6	0.45	3851734.9	14199.4	0.37

USP Tailing	0.88	0.00	1.38	1.10	0.01	0.76
Plate Count (USP)	3992	4.16	0.61	2592	18.37	0.71

SD: Standard deviation, %RSD:relative standard deviation

Specificity

The selectivity of the assay technique was determined by evaluating a peak profile as well as by analyzing the individual peak chromatogram, as presented in Fig. 3. Peak purity analysis was done using a photodiode array (PDA) detector, which illustrated that the analytehas been possessed an excellent chromatographic peak with no substantial interference or co-elution of impurities and degradation products.

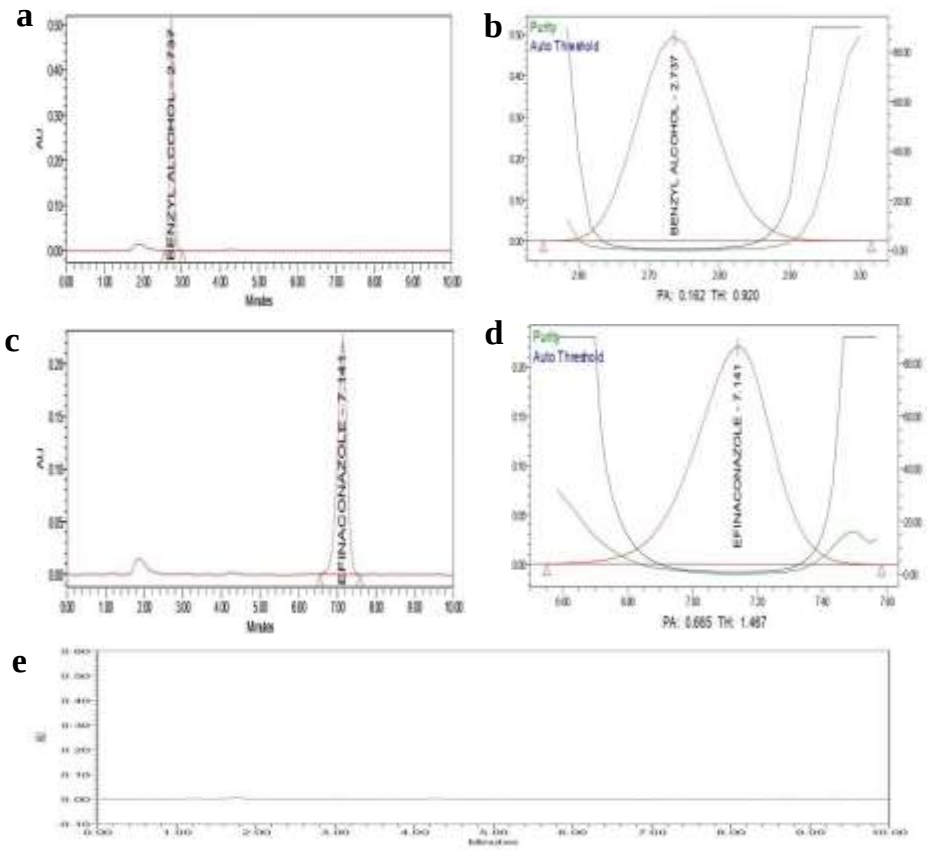


Fig. 3. Chromatogram of individual standard solution (a) peak ID of benzyl alcohol (b) peak purity of benzyl alcohol (c) peak ID of efinaconazole (d) peak purity of efinaconazole and (e) placebo chromatogram

The individual peak chromatogram was showed a single sharply defined analyte peak with no interference from other peaks of excipient or other components of the sample solution. Furthermore, we also ensured that the retention time of the analyte matched with standard as well as noted the absence of any other peak within the chromatogram. Theobtained results were provided conclusive evidence that the method was found specific, and there crown interference from other substances; hence, the analyte content was observed precisely quantified. The absence of other peaks and the constant retention time again went a long way in establishing the efficiency of the method, in terms of the purpose for which it was to be used, that is, the identification and measurement of the analyte of interest.

Solution Stability

The stability ofsample and standard solutions was evaluated over a 24-hours period at 10°C, and the % cumulative RSD was found to be less than 2.0% for both solutions. It showed that the solutions were above the calibration range during the time frame thus validating the HPLC method that was used to compare EFI and BA. The low % cumulative RSD value means that the solutions did not degenerate over the period which is important in ascertaining accurate and reliable results were obtained. The above findings validated the reliability of the method over the 24-hours period, since the peak areas of both sample and standard solutions remained consistent. It was confirmed that the sample solutions has not been degraded or

fluctuated in concentration. These outcomes supported the accuracy of the analytical strategy, ensuring the dependency & reliability of the outcomes, delivered through the HPLC analysis, strengthening the confidence in the total effectiveness of the methodology.

Linearity (calibration curve)

The concentration range for the linearity evaluation was spanned from 20% to 150% of the target concentration. For BA, this corresponded to a range of 16ppm (20%) to 120ppm (150%), while for EFI, the range was from 40ppm (20%) to 300ppm (150%). Standard solutions of both analytes were examined using the developed method, and the findings were utilized to evaluate the response of linearity. The values of R^2 determined were found 0.99996 for EFI and 0.99992 for BA, indicating an excellent linear relationship between the concentration of the analyte and the peak area. These high R^2 values confirmed the fact that the method has been found highly reliable when used for the quantification of both BA and EFI. The regression equations obtained where y = 18322.36564x + 2817.70509 for EFI and y = 47507.63548 x + 40862.69012 for BA (in Table 2) proved that the method used here is accurate and consistent. The linearity of the calibration curve can also be seen graphically in Fig. 4 and the correlation coefficient calculated from the data points substantiated the accuracy of the method over the whole concentration range. This robust linearity supports the reliability of the method for both analytes in routine quantitative analysis.

Table 2.Linearity, LOD and LOQ findings of efinaconazole and benzyl alcohol

Validation parameters	Efinaconazole	Benzyl Alcohol
Range (µg/ml)	40-300	16-120
Regression equation	y = 18322.36564x+2817.70509	y = 47507.63548x+ 40862.690
Slope	18322.36564	47507.63548
Correlation coefficient (r^2)	0.99996	0.99992
LOD (ng/ml)	507.49	2838.43
LOQ(µg/ml)	1.54	8.60

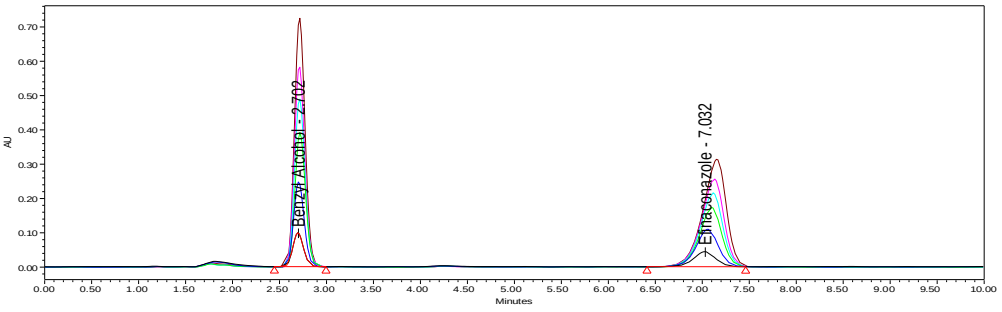


Fig. 4. a.) Calibration curves of efinaconazole b.) Calibration curves of benzyl alcohol and c.) HPLC overlay chromatogram of benzyl alcohol and efinaconazole

Detection and quantitation limits (sensitivity)

LOD and LOQ for efinaconazole (EFI) and benzyl alcohol (BA) were calculated using the standard deviation method reported in the experimental section. The calculated LOD for EFI was 507.49ng/ml, and the LOQ was 1.54 µg/ml. For BA, LOD was 2838.43ng/ml, and LOQ was 8.60 µg/ml. These results demonstrated that the method was delicate enough to identify EFI and BA at low concentrations, while also being capable of accurately quantifying them at higher concentrations (refer to Table 2). The ability to measure both analytes across a broad concentration range ascertained the reliability and versatility of the method for their analysis.

Accuracy

The recovery of the analytical procedure was assessed by spiking in placebo solution with an additional drug standard.The results showed that the recovery was found to be ranged from 98.0% to 102.0%. It was indicated that the method has been found highly effective in recovering the analyte, with no significant interference or loss during the process. The recovery data, along with the corresponding % RSD values, are summarized in Table 3. The % RSD remained consistently below 2%, which was well within the acceptable limit for method precision, highlighting the reliability of the method in repeated analyses. The low % RSD further depicted that the method had high degree of repeatability and low degree of variability, which is necessary for achieving high precision in the results when the method is run several time. The observed recovery values within the range of 98.0% to 102.0% further confirmed that the method has been found both precise and accurate for quantifying the analyte in the presence of other substances. Overall, these results validated the appropriateness of the implemented method for reliable and accurate quantitative analysis.

Table 3. Findings of Accuracy at different levels

Parameters	Efinaconazole	Benzyl alcohol
Accuracy at 80%	100.96	100.13
Accuracy at 100%	100.31	99.37
Accuracy at 120%	99.92	99.73
Average	100.39	99.74
SD	0.62	0.57
%RSD	0.61	0.57

SD: Standard deviation; %RSD: relative standard deviation

Precision

The system precision results demonstrated that the % RSD for the peak area of benzyl alcohol (BA) and efinaconazole (EFI) was 0.37% and 0.45%, respectively, which is within the acceptable criterion. Additionally, for the first injection of the standard solution, the theoretical plate count and tailing factor were 2574 and 1.10 for BA, and 3960 and 0.88 for EFI, both meeting the required specifications. These results indicated that the system has been provided consistent and reliable performance during routine analyses. In terms of method precision, the % assay for allsamples preparations was within the specification limit, with the % RSD for BA and EFI content across six sample preparations being 0.49% and 0.46%, respectively, further validating the method precision. For intermediate precision, the % assay values for all sample preparations, tested on several days, using various HPLC systems and columns, all met the specified limits. The % RSD of the assay results across six sample preparations was 0.48% for BA and 0.68% for EFI. The overall % RSD for replicate test sample preparations (considering both intermediate precision and method), was 0.47% for BA and 0.61% for EFI, demonstrating minimal variability and confirming the robustness of method. These results suggested that the method has been found rugged, performing consistently despite variations in variables likedistinct HPLC systems, columns, and test days. These findings, summarized in Table 4, supported the precision and reliability of method in analytical settings.

Table 4. Findings of method precision, intermediate precision and ruggedness of HPLC assay for efinaconazole and benzyl alcohol

Parameters	Efinaconazole		Benzyl alcohol	
S.No.	Method precision	Intermediate precision	Method precision	Intermediate precision
1	99.94	100.87	100.22	100.27
2	99.84	100.30	100.51	99.64
3	99.45	99.42	99.62	100.01
4	98.99	100.85	99.50	100.61
5	100.33	99.31	100.72	100.98
6	99.66	100.35	100.31	100.58
Average	99.70	100.18	100.15	100.35
STDEV	0.46	0.68	0.49	0.48
%RSD	0.46	0.68	0.49	0.48
Overall Average	99.94		100.25	
Overall STDEV	0.61		0.47	
Overall %RSD	0.61		0.47	

Degradation studies

Forced degradation (FD) studies (including Photolytic, thermal, acidic, basic and oxidation conditions) were performed on EFI and BA to assess their stability under various stress conditions, as outlined in Table 5. The reasonbehind this investigation was to explore the possible degradation based mechanisms and determine the chemophysical and photolytical stability of these drugs. As for the effects of chemical stress, it was observed that both EFI and BA had some compromise in their properties under conditions of acidic (1N HCl) basic (1N NaOH) and peroxide (3% H_2O_2) environment. Hydrolysis, induced by acidic and basic environments, resulted in degradation rates of 0.60% and 1.90% for EFI, and 0.70% and 0.92% for BA, respectively. Oxidative degradation induced by peroxide led to the highest degradation rates, with 3.01% for EFI and 1.26% for BA. These results suggested that both drugs are sensitive to hydrolytic and oxidative conditions. Regarding the physical demographical, both drugs were exposed to thermal stress where they were kept at 80°C for 24 hours. Comparing the two samples, the degradation rate of EFI was higher with a value of 1.09 % compared to BA with a value of 0.16% and therefore EFI is more sensitive to heat degradation as has been observed. On photolytic degradation, EFI did not decompose when exposed to dark for 7 days, but when exposed to direct sunlight, the degradation rate was 1.15% for EFI and 0.41% for BA, which showed that both EFI and BA are sensitive to photolytic degradation to some extent. The forced degradation studies were agreed with the observations in both scenarios; EFI stayed under 30% for total degradation. These results were found useful in understanding the causes of degradation of the four drugs and the need for the right storage and handling conditions. Further, the selectivity of the chromatographic method used in stability study of the compounds was established, which reaffirmed credibility of the method in terms of drug stability analysis. In sum, the findings of the study advance the general knowledge of the stability of the drugs that is critical to its safety and effectiveness.

Table 5. Results of degradation studies at different conditions shows peak purity

Parameters		Efinaconazole			Benzyl alcohol		
Sample name	Conditions	% Degradation	Purity Angle	Purity Threshold	% Degradation	Purity Angle	Purity Threshold
Control	NA	NA	0.592	2.240	NA	0.195	1.201
Photolytic Degradation	Direct sunlight for 7 days	1.15	0.890	2.357	0.41	0.340	1.412
Thermal Degradation	80°C for 24Hours	1.09	0.806	2.144	0.16	0.556	1.56
Acidic Degradation	1N HCl 80°C for 1Hour	0.60	1.012	1.928	0.70	0.569	1.063
Basic Degradation	1N NaOH 80°C for 1Hour	1.90	1.578	2.261	0.92	0.844	1.469
Oxidation Degradation	3% H_2O_2 80°C for 1Hour	3.01	1.143	2.178	1.26	0.441	1.298

Robustness

The robustness of the planned method was assessed by evaluating the sample and standard solutions of EFI and BA under various robustness conditions, as shown in Table 6 &7 and Fig. 5. Despite intentional variations in key parameters, the optimized techniques showed no remarkable changes in time of retention, factor of tailing and USP theoretical plates. Furthermore, system suitability variables were remained compliant under all tested robustness conditions, and the retention time for BA remained stable.

Changes in critical parameters, including flow rate (±10%), column oven temperature (±5°C), mobile phase composition (±10%), detection wavelength (±3 nm), and pH (±0.2), were tested to evaluate their impact on the performance of method. All variations met the criteria for acceptance, further confirming the method robustness to deliberate analytical adjustments. The results indicate that the method remains stable and reliable even under these variations, making it suitable for routine analysis without significant impact from changes in operational conditions. These findings affirm that the developed method is robust and can withstand typical variations encountered during routine analytical procedures. The various validations established here prove the strength and reliability of the method in achieving accurate quantification of EFI and BA, toward its applicability in pragmatic scenarios.

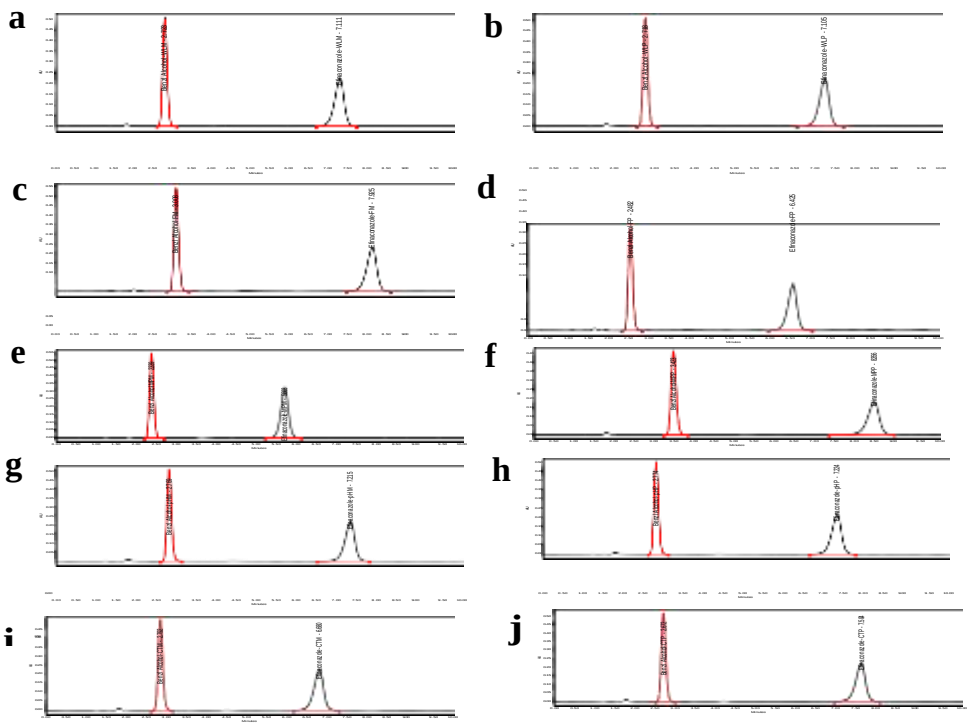


Fig. 5.Robustness chromatogram ofa.)wavelength minus chromatogram b.)wavelength plus chromatogram c.)flow minus chromatogram d.)flow plus chromatogram e.)mobile phase minus chromatogram f.)mobile phase plus chromatogram g.)pH minus chromatogram h.)pH plus chromatogram i.)column oven temperature minus chromatogram j.)column oven temperature plus chromatogram

Table 6.Findings of robustness for standard solution of benzyl alcohol and efinaconazole

Conditions	Standard solution of benzyl alcohol				Standard solution of efinaconazole			
	Peak Area	Retention Time	USP Plate Count	USP Tailing	Peak Area	Retention Time	USP Plate Count	USP Tailing
Ideal Condition	3869035	2.734	2608	1.10	3671071	7.147	3982	0.88
Wavelenght Plus	3193150	2.719	2678	1.10	2584581	7.105	4119	0.86
Wavelenght Minus	4226208	2.715	2678	1.10	4680974	7.104	4132	0.86
Mobile Phase Ratio Plus	4050930	3.406	3353	1.12	3590155	8.335	4100	0.83
Mobile Phase Ratio Minus	3971214	2.331	2265	1.08	4207582	5.591	4196	0.94
Flow Rate Plus	3606238	2.472	2442	1.09	3419446	6.414	3811	0.86
Flow Rate Minus	4430206	2.991	2995	1.12	4234263	7.911	4338	0.85
pH Plus	3865864	2.755	2767	1.11	3656081	7.206	4095	0.85

pH Minus	3864142	2.75	2731	1.10	3647030	7.198	4146	0.85
Column Oven Temp. Plus	3872790	2.666	2669	1.10	3723339	7.493	4439	0.85
Column Oven Temp. Minus	3883060	2.768	2715	1.11	3668996	6.655	3711	0.85

Temp. : Temperature

Table 7.Findingsof Robustness for the sample solution of benzyl alcohol and efinaconazole

Conditions	Sample solution of benzyl Alcohol				Sample solution of efinaconazole			
	Peak Area	Retention Time	USP Plate Count	USP Tailing	Peak Area	Retention Time	USP Plate Count	USP Tailing
Ideal Condition	3894885	2.726	2647	1.09	3699707	7.142	4018	0.87
Wavelength Plus	3161458	2.701	2641	1.10	2579956	7.089	4084	0.86
Wavelength Minus	4201548	2.706	2692	1.10	4670820	7.094	4135	0.86
Mobile Phase Ratio Plus	3993230	3.407	3349	1.13	3558149	8.339	4100	0.84
Mobile Phase Ratio Minus	3908623	2.322	2293	1.09	4166685	5.586	4179	0.95
Flow Rate Plus	3549183	2.465	2352	1.09	3397767	6.409	3792	0.87
Flow Rate Minus	4366400	2.991	3005	1.12	4155578	7.909	4406	0.84
pH Plus	3862136	2.758	2781	1.10	3667023	7.207	4095	0.85
pH Minus	3876485	2.753	2772	1.10	3671842	7.199	4129	0.84
Column Oven Temp. Plus	3867376	2.655	2706	1.10	3699157	7.488	4561	0.86
Column Oven Temp. Minus	3892773	2.775	27621	1.11	3702121	6.663	3718	0.85

Temp. : Temperature

Green aspects of recommended method

Analytical eco-scale (AES), Complex green analytical procedure index (ComplexGAPI), BAGI, software-driven analytical greenness index (AGREE), Analytical Greenness metric for sample preparation (AGREEprep) and Chloroform-Oriented Toxicity Assessment Scale (ChlorTax) are employed in distinct HPLC strategy. Any technique can be utilized to perform a detailed analysis to determine the degree of environmental consciousness of the product by evaluating its greenness profile.

The overall sustainability of the method received evaluation through the Eco-Scale analysis. The scoring system uses multiple variables to evaluate energy usage along with waste output in addition to chemical solvent toxicity levels during procedures. The method obtained an elevated score of **85** through penalty calculation which qualified it for the "superb green" classification(refer to Table 8 for detailed results). The scoring system emphasizes how environmentally sustainable this method is since it has received few penalties regarding waste output and chemical usage and energy usage [49]. The evaluation shows the method produces lower environmental impacts than standard drug testing methods thus qualifying as an environmentally friendly drug analysis solution.

The analytical protocol received full examination by using **ComplexGAPI** as a tool which evaluates all stages from pre-analysis procedures onward. The tool examines method performance through evaluation of fifteen principles from Green Analytical Chemistry (GAC) using a color scheme to grade practices with three hazard levels ranging from red as harmful to yellow as less harmful to green as environmental-friendly. Sustainability emerged as a vital factor because the tool categorized important principles relating to solvent usage and waste management both as green which represents minimal

harm to the environment(refer to Table 8). The steps that involved energy usage obtained yellow ratings indicating their impact on the environment could be enhanced to improve performance in upcoming versions [50]. ComplexGAPI evaluation showed that the analytical method demonstrates robust green chemistry compliance so it presents a useful approach for decreasing environmental damage in analytical chemistry fields.

An evaluation of method applicability and effectiveness used the **BAGI tool** which was founded on the ten fundamentals of White Analytical Chemistry. The analytical method achieved an assessment total of 85 points which demonstrated its strong compatibility with principles of sustainable analytical efficiency(refer to Table 8 for detailed results). The method demonstrates effective performance for solvent utilization as well as waste reduction and energy management according to this assessment score [49, 50]. Modern analytical practice involving environmental responsibility can use this method judging from its positive outcome since it demonstrates its capacity to serve eco-friendly drug analysis.

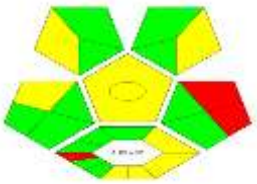
The **AGREE (Analytical GREEnness)** tool delivered by Gdańsk University of Technology allowed for evaluating the environmental sustainability of the method. The method achieves impressive sustainable environmental characteristics based on its AGREE score of **0.81** which places it in the "excellent green performance" category (0.8–1.0)(refer to Table 8). The method demonstrates excellent sustainability characteristics because its waste reduction measures and energy utilization improve while retaining environmentally benign solvents. The method demonstrates potential framework for sustainable replacement of traditional analytical methods through its positive AGREE assessment result.

Through **AGREEprep** the developers gained specific sustainability information about how effective the method was for preparing samples. The method achieved a rating of 0.67 which classified it under the "good environmental performance" group. The method demonstrates good sustainability and efficient operation during sample preparation though it requires optimization of energy usage and solvent management(refer to Table 8). According to the AGREEprep evaluation the method shows a satisfactory environmental performance although its researchers note that enhancements in sustainability can be achieved through better refinements.

The final measurement tool used was Chloroform-Oriented Toxicity Assessment Scale (ChlorTox) for assessing chemical risks of the technique. The chemical risk assessment with ChlorTox Scale determines toxicity by combining the ChlorTox values of all chemical reagents which represent chloroform toxicity equivalents. The ChlorTox analysis showed that the procedure method poses less chemical hazard risk compared to methods which use toxic ingredients(refer to Table 1 for detailed results). Testing confirmed the method creates minimal environmental threats and health risks hence making it suitable for drug analysis as both environmentally safe and health-friendly alternative [49].

These various assessments confirm that the newly developed HPLC method shows high sustainability through green chemistry principles while reflecting minimal environmental impact. The method proves advantageous for environmental analytical practices because of its efficient waste reduction alongside minimal energy use together with its sustainable reagent choice. The analyzed results show that this method presents strong potential to minimize environmental damage and chemical safety hazards in analytical chemistry practice.

Table 8. Assessment of Greenness parameters

Tools for Assessment	HPLC Method
Eco Scale Assessment	Methanol – 4.0
	Instrument – 3.0
	Energy employed – 0.0
	Waste – 5.0
	Occupational hazard – 3
	Total penalty points –∑15
	Analytical eco-scale – 85
ComplexGAPI	
BAGI	
Agree	
Agreeprep	
ChlorTox scale	**Methanol**
	Relative hazard (WHN) – 0.57
	Relative hazard (CHEMS-1) – 0.15
	M sub [mg] – 5.27
	ChlorTox (WHN) [g] – 3.005
	ChlorTox (CHEMS-1) [g] – 0.79

Application of the method

An RP-HPLC method, ascertaining it to comply with ICH guidelines, enclosed critical factors like linearity,selectivity, precision, accuracy, robustness, specificity, LOD and LOQ,specifically tailored for quantitative determination of benzyl alcohol and efinaconazole.Thisprocedure has beencharacterized and confirmed to be specific, sensitive, accurate, simple and

rapid in its capability to measure both BA and EFI levels accurately.It has been successfully applied to measure the drug content in pharmaceutical dosage forms, meeting a variety of validation criteria. The method was utilized to assess the levels of BA and EFI in NLC gel formulations containing 1% w/w efinaconazole. After drug extraction, all sampleswere measured three times according to the method mentioned in sample preparation section. Both the increases of percentage recovery of EFI and BA in the gel were 100.12% and 99.74% respectively. More significantly, the peaks of reaction analytes in both Figures 6(a) and 6(d) did not have any other compound interfering with them. The authenticity of the highest molecular ion peaks of BA and EFI was further verified by comparing the spectra of the compounds, isolated from the gel, with the spectra of the standards BA and EFI (Figures 6(e) and 6(f)]. This method was optimised and has been shown to be fast, sensitive, sequential, and selective for the determination of BA and EFI. The apparatus was observed to have a short total analysis time of 10 minutes thus making it suitable for frequent sampling in pharmaceutical formulations. The choice of the approachesconcerning main features, which include cost as well as simplicity of the proposed method as well as compatibility of the new method with the existing equipment, solvents, and time required for analysis, has been also carefully discussed, and thus, the method can be employed for regular testing.

The quantitative measurements and assay results of this method have been found to be matched the intended acceptance level for both EFI and BA, proving that this method offers reliable and effective results. When it comes to samples from accelerated stability studies and general formulation testing, the method has been worked just as well. Moreover, it has also been observed to determine the degradation rate of products under specific conditions and in formulations, and thus enhance the quality and safety. These stability tests further showed that EFI was generally more sensitive towards oxidation whilst no disturbance in the chromatograms was observed. These findings substantiate the method’s selectivity and appropriateness for estimating EFI and BA to improve formulation safety and quality. It is evident from these outcomes, that the proposed method efficiently estimates BA and EFI in their mixture preparation at optimum, and analytically comparable performance. In addition, the method was validated, and hence, its potential for future use in bioanalysis applications.

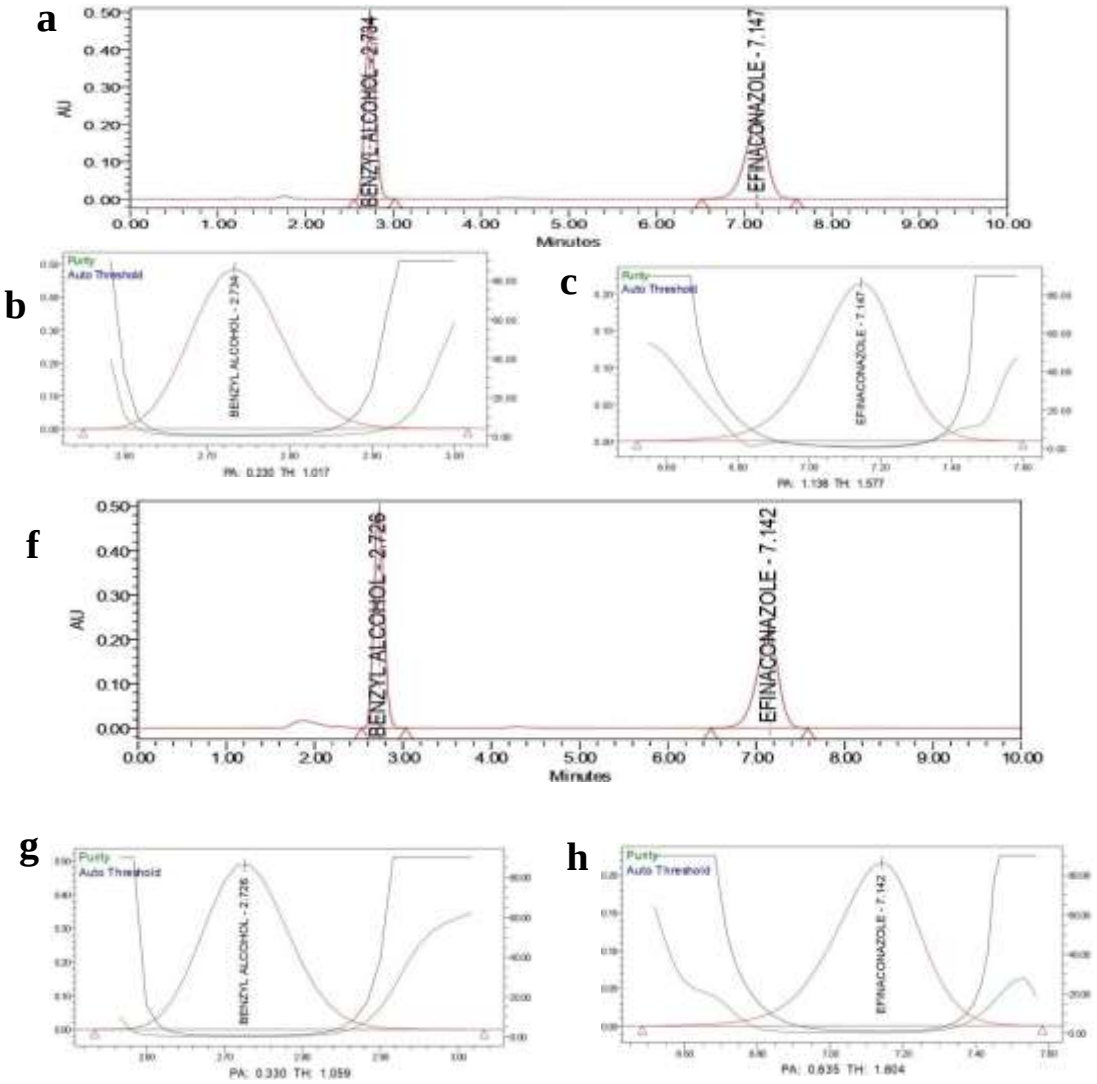


Fig. 6. Analyte peaks a.) standard solution chromatogram of benzyl alcohol and efinaconazole, b.) peak purity of benzylalcohol in standard solution, c.) peak purity of efinaconazole in standard solution, d.) NLC-GEL sample solution chromatogram of benzyl alcohol and efinaconazole, e.) peak purity of benzylalcohol in sample solution, and f.)peak purity of efinaconazole in sample solution.

4. CONCLUSION

Researchers have developed a variety of analytical techniques for the separate measurement of benzyl alcohol an efinaconazole. Herein, the developed RP-HPLC method for the simultaneous estimation of benzyl alcohol (BA) and efinaconazole (EFI) demonstrates to be precise, accurate, and reliable. The developed method also showed good linearity for both the compounds with corresponding coefficients of correlation up to the required concentration for both the compounds and thus providing reproducible analysis. The LOD and LOQ were set appropriately to enable the measurement of desired sensitivity and accuracy in the presented analysis. Therefore, method validation in accordance with ICH guidelines demonstrated its authenticity as well as the assay's variability parameter was in compliance with the permissible rein at 0.29%. Therefore, this method can be most appropriate and reliable for routine estimation of BA and EFI in developed formulations as well as quality assurance in the pharmacological field. The validation carried out has been involved various parameters such as robustness, accuracy, quantification limits, detection limits, linearity, precision, specificity and stability indicating capacity without much compromise. Stress test performed according to different conditions met the previously defined acceptance, which testifies to the effectiveness of the method in analyzing drug samples.

The percent recoveries of standard and sample solutions remained stable for at least 24 hours at 10°C during solution stability tests which showed that the technique had both accuracy and endurance throughout extended time duration. The present method proves itself capable of being an efficient stability-indicating solution for sustained database accuracy of EFI & BA throughout pharmaceutical formulation shelf-life. Numerous pharmaceutical quality control laboratories utilize this method to assess EFI stability together with BA drug potency because it ensures safe incorporation of these compounds into multi-formulation drug products. The method development relied on multiple environmental tools which include AES, ComplexGAPI and AGREE to demonstrate its focus on minimizing environmental effects. The tools establish compliance of the method with present sustainability requirements for analytical procedure standards. The assessment method provides an essential capability to examine the stability and potency of both BA and EFI along with guaranteeing drug formulation safety to establish itself as an important analytical solution for pharmaceutical stability tests and quality control.

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Conflicts of interest

The authors declare no competing interests.

Ethical approval

As this study does not involve animal and patient experiments, the ethical approval and consent to participate are not applicable.

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