ACS OMEGA

Open Access

http://pubs.acs.org/journal/acsodf

Article

Simultaneous Determination of Posaconazole and Hemp Seed Oil in Nanomicelles through RP-HPLC via a Quality-by-Design Approach

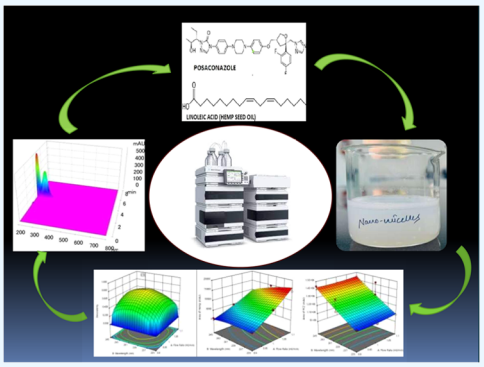
Anjali Rathee, Pavitra Solanki, Surajpal Verma, Divya Vohora, Mohammad Javed Ansari, Alhussain Aodah, Kanchan Kohli, and Yasmin Sultana*

Cite This: *ACS Omega* 2023, 8, 30057−30067

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: The present study involves the development of a reverse-phase HPLC method employing the quality-by-design methodology for the estimation of posaconazole and hemp seed oil simultaneously in nanomicelles formulation. The successful separation of posaconazole and hemp seed oil was achieved together, and this is the first study to develop and quantify posaconazole and hemp seed oil nanomicelles with linoleic acid as the internal standard and developed a dual drug analytical method employing a quality-by-design approach. The study was performed on a Shimadzu Prominence-I LC-2030C 3D Plus HPLC system with a PDA detector and the Shim-pack Solar C8 column (250 mm × 4.6 mm × 5 μm) for analysis with a mobile phase ratio of methanol:water (80:20% v/v) maintaining the flow rate of 1.0 mL/min. The final wavelength was selected as 240 nm and the elution of hemp seed oil and posaconazole was obtained at 2.7 and 4.6 min, respectively, with a maximum run time of 8.0 min. Box Behnken design was employed to optimize the method, keeping the retention time, peak area, and theoretical plates as dependent variables, while the mobile phase composition, flow rate, and wavelengths were chosen as independent variables. Parameters such as specificity, accuracy, robustness, linearity, sensitivity, precision, ruggedness, and forced degradation study were performed to validate the method. The calibration curves of posaconazole and hemp seed oil were determined to be linear throughout the range for concentration. The suggested approach can be effectively utilized for estimating the content of drugs from their nanoformulation and proved suitable for both *in vivo* and *in vitro* research.



INTRODUCTION

Despite the reality that many fungal strains have inhabited the environment, about 500 species are considered opportunistic pathogens in humans. Many fungal species found in nature have been a component of regular flora living in animals and people. Infections caused by fungi may vary from frequent, moderate, and superficial to fatally invasive diseases, particularly among patients with weak immune systems. Almost bulks of invasive fungal infections (IFIs) are associated with higher morbidities and deaths in immunocompromised patients suffering from cancer, HIV, transplantations, infants, and individuals.[1] It is estimated that billions of people worldwide get affected by harmful fungi, with about 1.5 million casualties occurring annually.[2] The persistent likelihood of IFIs as well as the considerable mortality associated with fungal diseases in immunocompromised patients necessitates the use of dual antifungal medication.

Posaconazole is an antifungal agent of the triazole category, shown in Figure 1a,[3] produced through itraconazole, shown in Figure 1b,[4] which performs similar functions by lanosterol enzyme inhibition and hampering ergosterol biosynthesis required by the membrane of fungal cells like different azole analogues. This drug is approved for the treatment of IFIs in patients suffering from oropharyngeal candidiasis, coccidioidomycosis, fusariosis, mycetoma, and chromoblastomycosis.[5] It is also given as a prophylactic medication in acute myeloid leukemia patients undergoing induction chemotherapy and recipients who receive treatment for immunosuppression after hematopoietic stem cell transplant (HSCT) along with myelodysplastic syndrome (MDS) patients with a risk of IFI development due to long periods of neutropenia.[5] Posaconazole is highly suggested for the therapy of mucormycosis in combination with amphotericin-B (AmB) for the treatment of refractory illnesses or in cases of resistance to earlier medications.[6] *In vitro* and animal studies have been performed to observe the synergistic action of posaconazole coupled with either micafungin or caspofungin.[7] Synergistic *in vitro* as well as

Received: March 29, 2023
Accepted: July 24, 2023
Published: August 11, 2023

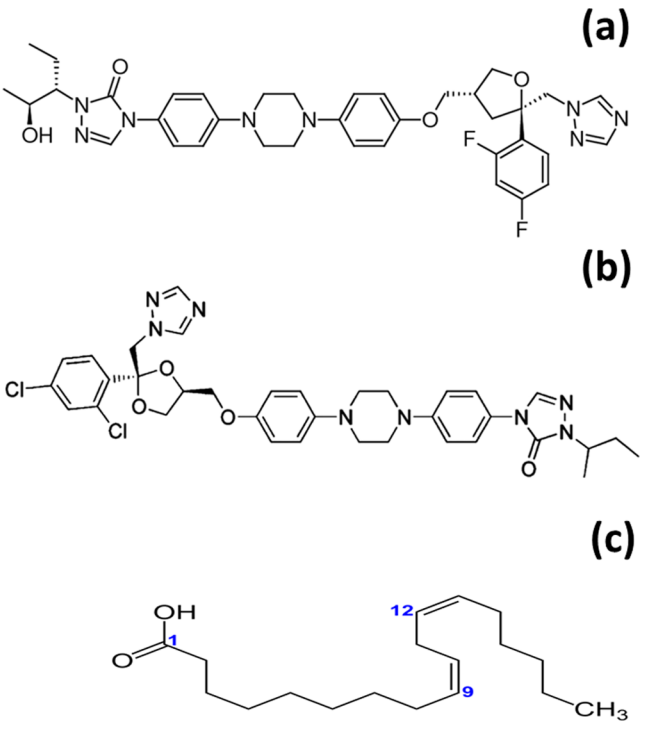


Figure 1. (a) Structures of posaconazole, (b) itraconazole, and (c) linoleic acid.

in vivo activities of posaconazole combined with caspofungin against *Candida albicans* have been reported.[8]

Cannabis sativa L., commonly known as Hemp is used widely for its nutritive, medicinal, and textile benefits as it contains oils, phytochemical compounds, and fibers.[9,10] The plant has become a promising source of therapeutic agents for its potent antioxidant activities.[11,12] It is used for the treatment of various ailments including eczema, psoriasis, osteoporosis, premenstrual syndrome, menopause, multiple sclerosis, diabetes, and body care. Although hemp seed oil has the presence of Δ-9-tetrahydrocannabinol (THC), one of the primary psychoactive constituents of marijuana, these are present in very minute quantities and are estimated to be less than 0.2%;[13,14] therefore, it does not require a specialist's certificate for use.[15] Hemp oil has been observed to contain a high proportion of antioxidant compounds and therefore aids in boosting the immune response and is also reported to have fewer adverse effects.[16]

Over the past decades, nanomicelles have sparked enormous attention toward the diagnosis and treatment of a variety of ailments, ending with FDA approval.[17] These are typically made from amphiphilic polymers that are formed in a solvent with a hydrophilic exterior and a hydrophobic reinforced layout of nanomolecules.[18] The presence of a lipid-soluble center increases water solubility, thus permitting controlled pharmaceutical delivery.[18,19] When prompted by stimuli like temperature or pH, they release medicines, resulting in improved specificity and lesser side effects.[18] During usual physiological conditions of pH 7.2, the medication remains contained within them, but during acidic conditions, the medicinal compounds are released in the specified portion of the body where treatment is required. The functions of the pH factor play an important role in triggering and causing the medicine to be released in the preferred location.[20] Additionally, their small particle size increases the period of stay in the bloodstream, hepatic passage, filtering via the spleen, renal elimination, cell uptake, as well as the ability to cross epithelium membrane boundaries.[21] Such elements all help to increase drug bioavailability.[22]

Nowadays, there is a wave in the universal trend of shifting from synthetic derivatives to herbal medicines, which is known as "Return to Nature". There are a lot of reasons for these natural products gaining interest because of their nontoxic nature, having fewer side effects, and being cost-effective.[23] The combination of synthetic drugs with phytoadjuvants can overcome the challenge of synthetic drug treatment as there is a reduction in the high doses of synthetic moieties when used alone, which further reduces the adverse effects, high costs of treatment, toxicity, and multidrug resistance and provides a synergistic and additive action, which can be further used for industrialization.[24] The usage of herbs counted as an important hand in conventional antifungal therapy because their active constituents in combination with an antifungal regimen are likely to reduce the minimum effective dose of the drugs and further provide a lot of benefits.[25]

The synergistic effects of posaconazole and hemp seed oil have not been studied previously. Hence, this study would provide a better understanding of their effects. The current study aims to develop, quantify, and validate posaconazole and cold-pressed hemp seed oil with its major constituent linoleic acid as shown in Figure 1c[26] for a dual drug analytical method and its validation through reverse-phase high-performance liquid chromatography (RP-HPLC).[27]

MATERIALS AND METHODS

Materials. Posaconazole was purchased from TCI Chemicals and the extracted hemp seed oil was obtained as a gift sample from Kumaokhand AIH Pvt., Ltd. Transcutol HP, PLGA, Tween 80, Precirol ATO 5, and poloxamer 188 were purchased from Sigma-Aldrich. The experiment was conducted using high-grade chemicals for HPLC.

Methods. *Instrumentation and HPLC Conditions.* The Shimadzu Prominence-I LC-2030C 3D Plus instrument manufactured in Japan was used for high-performance liquid chromatography. The analytes were distinguished through a 5 μm column (C8) with a length of 250 mm and width of 4.6 mm using a Shim-pack Solar, Shimadzu, Japan, in reverse phase (RP) under appropriate wavelengths of 230 nm for hemp seed oil and 262 nm for posaconazole. The method was developed at 240 nm and the maximum absorption of the analytes was observed. We used 80:20 ratios of methanol and water as the mobile phase composition, freshly prepared, degassed, and filtered using a nylon membrane of 0.22 μm before utilization. The temperature of the column was kept constant at 30°C and the rate of flow was 1 mL/min, with 8 min of run time.

Standard and Working Solution Preparation. Standard solutions were made for both the analytes (A1—posaconazole; A2—hemp seed oil) using methanol to obtain a concentration of 1000 μg/mL. 2 mL of the standard solution was taken and mixed in methanol to make stock B1 along with stock B2 by reaching a concentration of 200 μg/mL and further diluting it using methanol to create a blended working solution of stock in varying concentrations of 20, 40, 60, 80, 100, and 120 μg/mL accordingly for the analytes separately.[28] Moreover, the preparations of both the analytes were identically processed for quality control analysis after filtration of the solutions using 0.22 μm Milford Millifilter, MA.

https://doi.org/10.1021/acsomega.3c02097
ACS Omega 2023, 8, 30057−30067

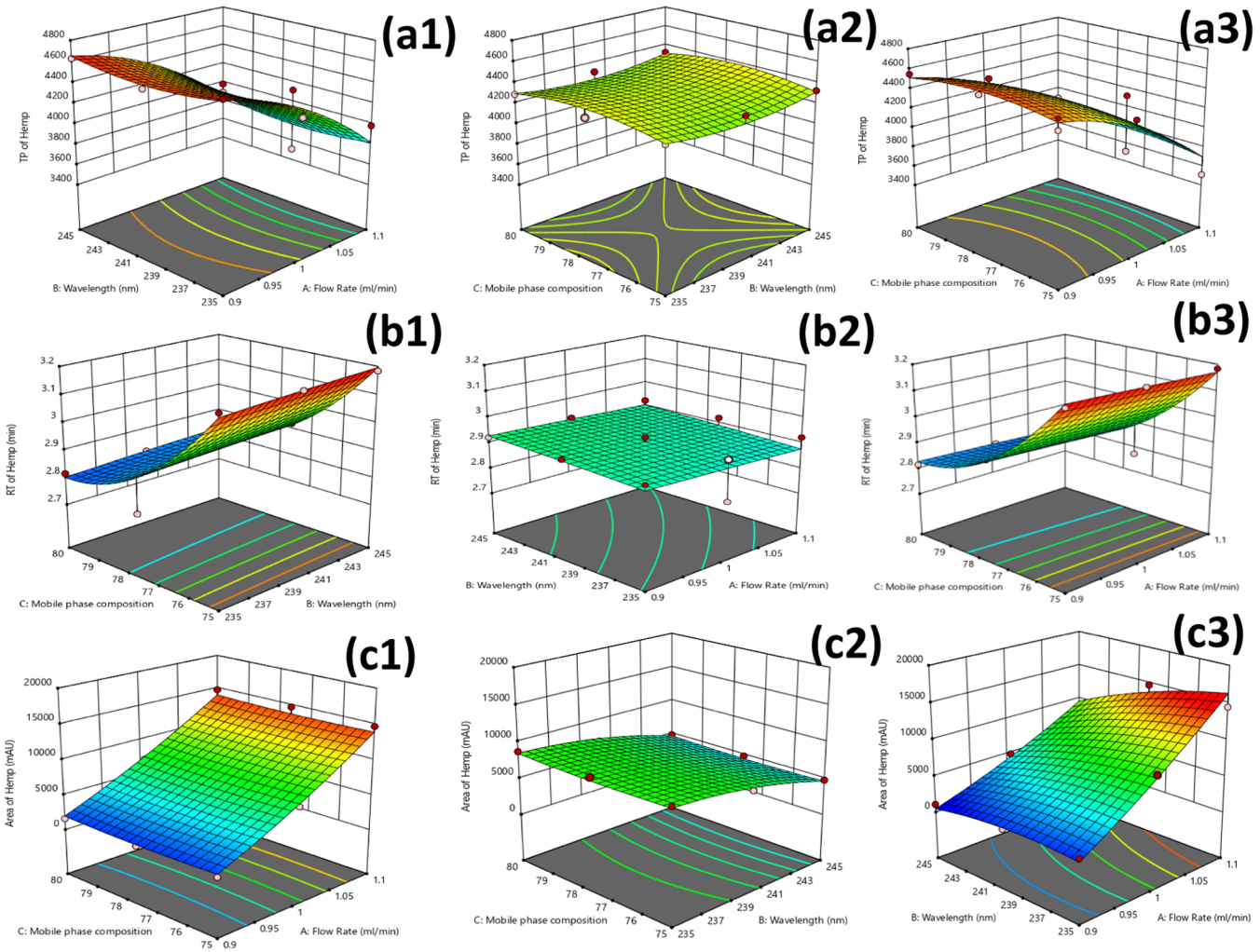


Figure 2. 3D response surface graphs illustrating the effect of flow rate (mL/min), wavelength (nm), and mobile phase composition, on (a1−a3) theoretical plates, (b1−b3) retention time, and (c1−c3) peak area of hemp oil.

RP-HPLC Method Optimization. Box Behnken designing was used to optimize the suggested technique, in which response variables were six, while factor variables were three. The independent variables tested were flow rate (A), wavelength (nm) (B), and organic solvent content in the mobile phase or mobile phase composition (C), while the dependent variables were posaconazole retention time (RT) ($Y1$); hemp seed oil retention time ($Y4$), posaconazole area ($Y2$), hemp seed oil area ($Y5$), posaconazole theoretical plates (TP) ($Y3$), and hemp seed oil theoretical plates ($Y6$). The Design Expert software of STATEASE Inc. was used to run 29 tests with polynomial formulas to examine the overall responses, Y (eq 1)

$$Y = \beta 0 + \beta 1A + \beta 2B + \beta 3C + \beta 12AB + \beta 13AC + \beta 23BC + \beta 11A2 + \beta 22B2 + \beta 33C2 \quad (1)$$

The terms used for the quadratic equation (eq 1) were AB, BC, AC, $A2$, $B2$, and $C2$. The independent variables A, B, and C denoted the flow rate, wavelength, and organic phase content in the mobile phase, respectively. The variable $\beta 0$ was the intercept, while $\beta 1$, $\beta 2$, and $\beta 3$ were linear coefficients. The coefficients for interaction were $\beta 12$, $\beta 13$, and $\beta 23$, while the quadratic coefficients were $\beta 11$, $\beta 22$, and $\beta 33$.

Analysis of variance (ANOVA) was used to verify the models' significance, while the response surface methodology (RSM) was utilized to establish the relationship between independent and dependent variables.

Validation of the Method. This methodological approach has been verified for linearity, specificity, robustness, ruggedness, suitability test of the system, precision, detection, and quantification limit. Stability was performed under various test conditions.[29]

System Suitability Analysis. System suitability analysis is an essential component of the liquid chromatographic procedure to ensure that the methodology and the entire system for testing are reproducible and appropriate for the desired purpose. The system suitability analysis was performed at 20 μg/mL moderate quality control level after injection replicates and the results analyzed using the peak area, retention time, and the column's theoretical plates. The volume of injection was 10 μL of posaconazole and 1 μL of linoleic acid. According to the guidelines of the United States Food and Drug Administration, the system suitability analysis requires that the relative standard deviation (% RSD) of the responses (peak area, retention time) should be less than 2%. Similarly, the theoretical plates of the column should be more than 2000.[28]

Specificity. An important feature of HPLC is the specificity that relates to the system's analytical capacity to extract

https://doi.org/10.1021/acsomega.3c02097
ACS Omega 2023, 8, 30057−30067

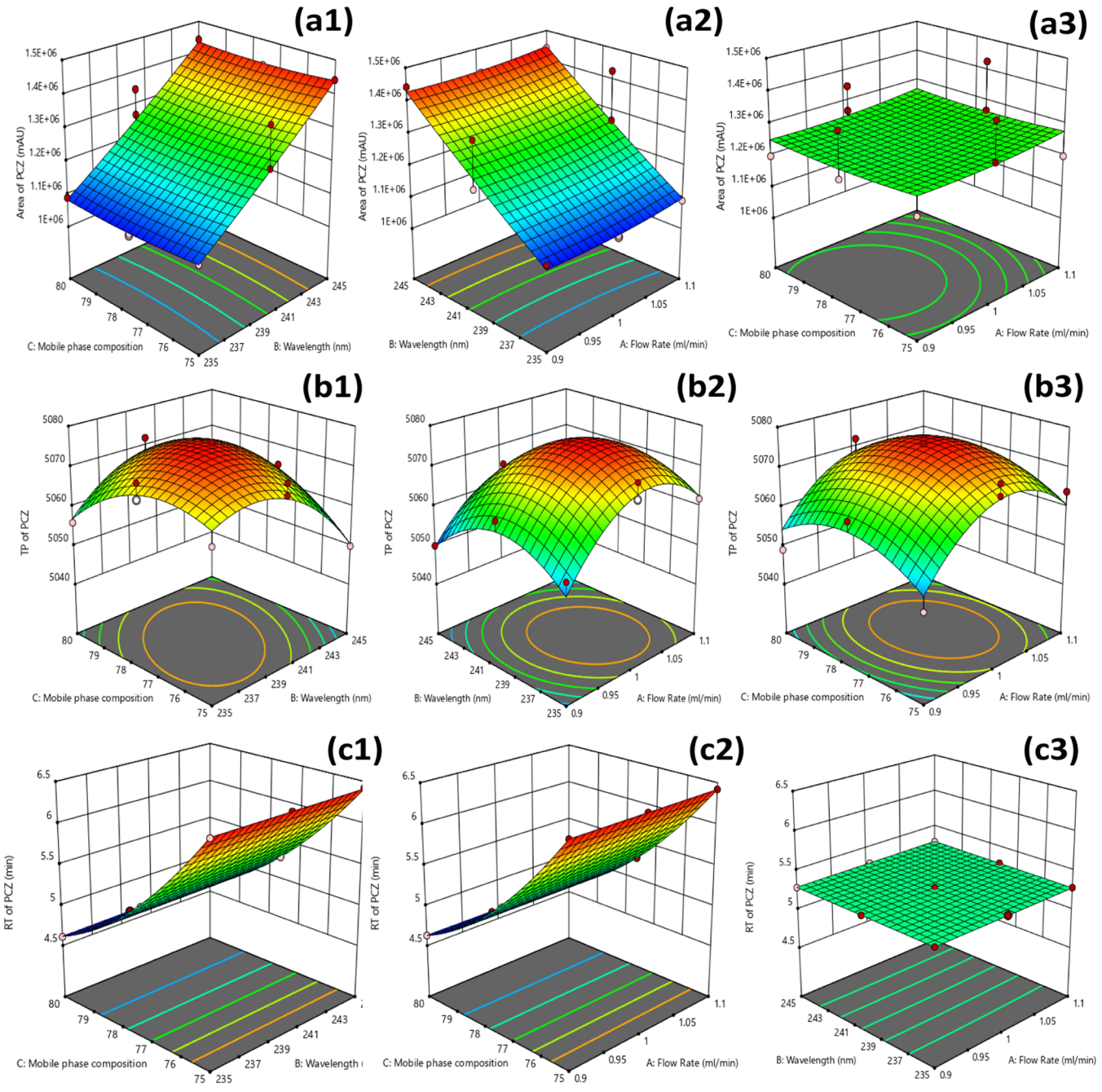


Figure 3. 3D response surface graphs illustrating the effect of flow rate (mL/min), wavelength (nm), and mobile phase composition, on (a1−a3) peak area, (b1−b3) theoretical plates, and (c1−c3) retention time of posaconazole.

analytes from complicated samples. It is determined through the comparison of chromatograms of single solutions and their samples and a blank solution without posaconazole and hemp seed oil at levels of moderate quality control.

Linearity. Different strengths of mixed solutions of standards containing concentrations of the target at 100, 200, 300, 400, 500, and 600 μg/mL were taken to observe hemp seed oil, while the concentrations of 20, 40, 60, 80, 100, and 120 μg/mL were taken for posaconazole. The percentages were created accordingly to evaluate linearity. Separate curves for posaconazole and hemp seed oil calibration were drawn with an area of the peak on the y-axis and specific concentrations on the x-axis. The equations for regression were computed along with the response factor by simple division of the area of peak by concentration.

Sensitivity. The analytical method's sensitivity was calculated using detection limit (LOD) because the concentration offers a signal-to-noise proportion of 3:1 and quantification limit (LOQ) as it produces a signal-to-noise of around 10:1 with lower than 10% RSD. To calculate the detection and quantification limit, the formula used was $A = k\sigma/S$ where A is the detection limit or quantification limit, σ is the standard deviation (SD) of peak area response, and S depicts the slope observed through the curves of calibration. The value of k for the detection limit was 3.3 and the quantification limit was 10.

Robustness. The test was carried out to evaluate the method's appropriateness by varying chromatographic con-

https://doi.org/10.1021/acsomega.3c02097
ACS Omega 2023, 8, 30057−30067

ditions. The column temperature was varied from 25 to 35 °C and the wavelength of detection of posaconazole and hemp seed oil was varied from 235 to 245 nm. 100% of the concentration target at 100 μg/mL moderate quality control level was injected in triplicates for each chromatographic condition stated above. The % RSD of the average area of the peak along with the percentage of recovery of posaconazole and hemp seed oil was used to assess the method's robustness. Its optimum level must not be higher than 2%.[28]

Accuracy. The test method's accuracy is defined as the closeness between the predicted and discovered values. The suggested method's accuracy was assessed by the percentage recoveries of the analytes at quality control levels of 50, 100, and 150 % with three replicates of the sample to be injected per concentration. The standard error (SE) and mean percentage recoveries of posaconazole and hemp seed oil following the % RSD were evaluated using the formula given below (eq 2)

$$\%\ \text{recovery} = [((\text{concentration recovered}) / (\text{concentration injected})) \times 100] \quad (2)$$

Precision. The measurement of precision for the method was to assess numerous replicates in distinct scenarios. The method's precision for the interday test was evaluated by analyte samples of quality control with three replicates of each drug sample for 3 continuous days and the same day as well. The analyte's area of peak and percentage of recovery were observed, and calculation of % RSD with values less than 2% along with the retention time, area, and column theoretical plates was done.[30]

Preparation of Posaconazole−Hemp Seed Oil Nanomicelles. The nanoprecipitation approach was used to create posaconazole and hemp seed oil nanomicelles using PLGA.[31] Furthermore, a 1% solution of PLGA was made by the dissolution of acetone and the polymer PLGA (50:50). Different amounts of posaconazole and hemp seed oil were dissolved in 1% acetone and PLGA solution (5 mL) in three distinct solutions with ratios of 15:1, 10:1, and 5:1 after careful assessment of acceptable surfactant concentrations. The organic phase was mixed in an aqueous medium (50 mL) containing poloxamer 188 at a specified concentration of 0.3 mL/min while stirring at a constant speed. The final formulation turned milky immediately due to the formation of nanomicelles. After 6 h of evaporation at 60° C, approximately 30 mL of posaconazole−hemp seed oil nanomicelles formulation was obtained.[32−34]

Stability Studies. Studies for stability were carried out to evaluate solutions at different quality control measures and in various circumstances such as the time duration of the freeze−thaw cycle. Aliquots of samples were tested for thaw at 37 °C unaided and were frozen for a day at −20 °C. To observe stability for a short duration, the quality control sample was kept at 37 °C and tested after 4 and 12 h. Stability tests for the long duration were conducted at −20 °C for 14 days. Moreover, the analyte solutions of the standard were tested for 6 h at 37 °C and 14 days at −20 °C. These tests were performed three times and were evaluated by the percentage of recovery and mean SD within an appropriate range.

RESULTS AND DISCUSSION

Development and Optimization. The design of Box Behnken was effectively used to optimize and yield the experimental 3D graphs that depict the influence of factors on response variables. The projected values of r^2 agreed rather well with the amended value of r^2. Furthermore, the large values of the amended r^2 demonstrated a strong connection between the fitted model and the data obtained from the experiment. Equations of polynomials were utilized to determine the real relationship between the responses and the variables. The response obtained was a positive value denoting an impact that supports optimization, while a negative value denotes an inverse connection between the variables. Through the quadratic equation, the values observed were negative in dual terms (*AB* and *BC*) of interaction, while the interaction of *AC* produced a positive result of the *Y*1 response.

Table 1. System Suitability Parameters of Posaconazole and Hemp Seed Oil

system suitability parameters	retention time (min)	peak area (mAU)	theoretical plates (N)	tailing factor
posaconazole	4.65	289,173	5072	1.06
	4.66	289,669	5064	1.06
	4.66	289,500	5070	1.06
mean	4.66	289,447.30	5068.70	1.06
SD[a]	0.005	252.15	4.16	0.002
RSD[b]	0.12	0.08	0.08	0.19
hemp seed oil	2.77	1649	4517	1.21
	2.78	1620	4517	1.22
	2.77	1680	4530	1.23
mean	2.77	1649.66	4521.33	1.22
SD[a]	0.005	30.005	7.503	0.009
RSD[b]	0.19	1.81	0.16	0.74

[a]Standard deviation. [b]Relative standard deviation.

Figure 2a1−a3,b1−b3,c1−c3 depicts the influence of independent variables (*A*—flow rate, *B*—wavelength (nm), and *C*—organic solvent content in the mobile phase (mobile phase composition)) on the theoretical plates, retention time, and area (mAU) of hemp seed oil, respectively. Similarly, Figure 3a1−a3,b1−b3,c1−c3 depicts the influence of independent variables (*A*—flow rate, *B*—wavelength (nm), and *C*—organic solvent content in the mobile phase (mobile phase composition)) on the theoretical plates, retention time, and area (mAU) of posaconazole, respectively. The model *F*-value of 8.16 was obtained for posaconazole, which implied that the model was significant.

As depicted in Figure 2a1−a3, the hemp oil produced the following changes on theoretical plates as described. A significant effect was seen on the theoretical plates upon changing the flow rate. As the flow rate increased, the theoretical plates were reduced. In the case of mobile phase composition, the theoretical plates were increased initially but reduced as the composition increased to 80:20. On the other hand, wavelength also had a similar significant effect. As the wavelength increased from 235 to 240, a decreasing trend in the theoretical plates was observed although the TP of hemp starts increasing when the wavelength was increased to 245.

As observed in Figure 2b1−b3, the hemp oil produced the following changes in retention time as described. As the flow rate was increased, the retention time was slightly reduced. Similarly, RT was also shifted slightly upward after increasing the wavelength. On the other hand, RT was reduced drastically after increasing the mobile phase composition, indicating the most significant effect of composition on the RT of hemp.

https://doi.org/10.1021/acsomega.3c02097
ACS Omega 2023, 8, 30057−30067

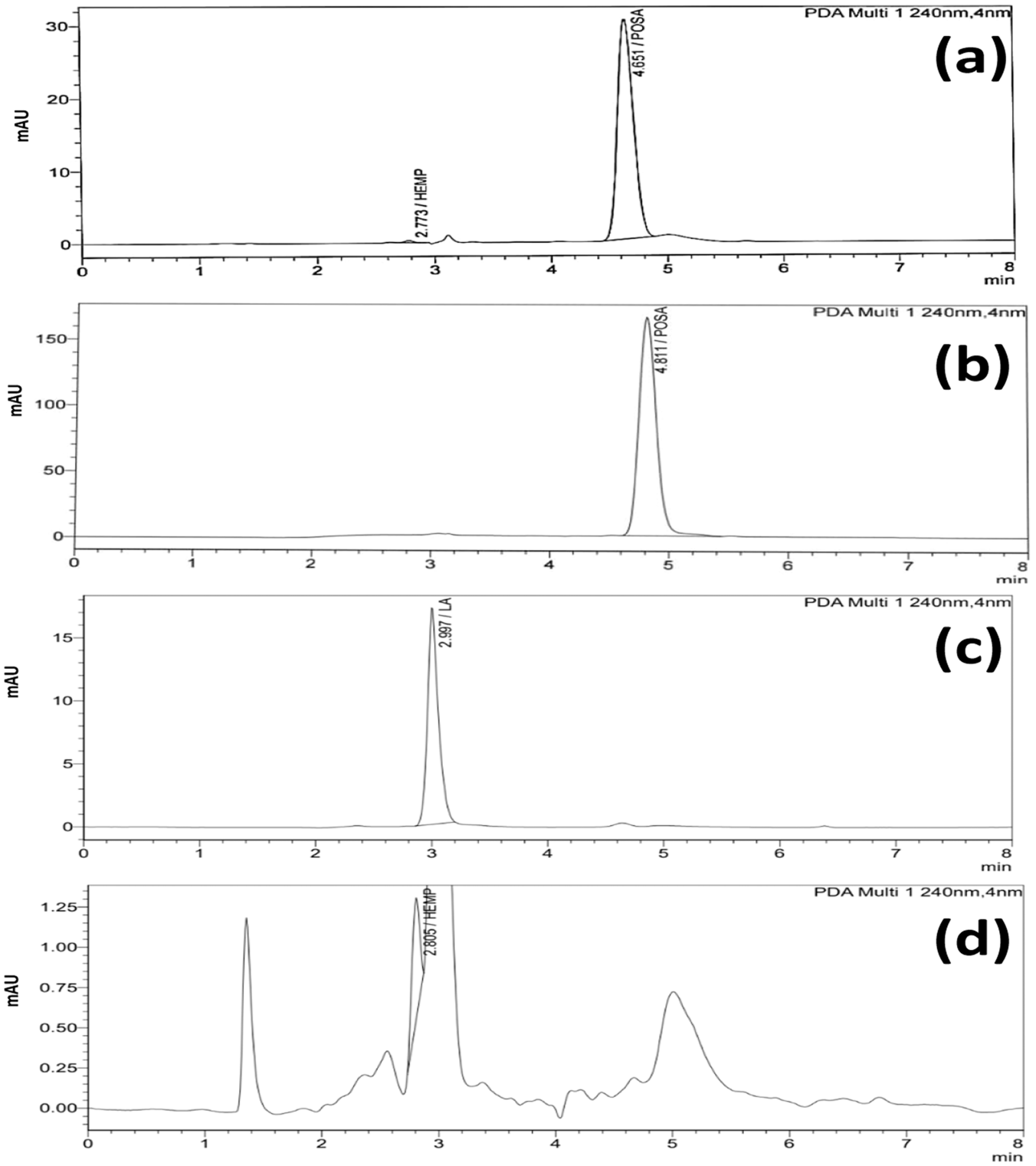


Figure 4. (a) Chromatogram of a combination of hemp seed oil and posaconazole, (b) posaconazole, (c) linoleic acid (IS), and (d) hemp seed oil.

As depicted in Figure 2c1−c3, the hemp oil produced the following changes in the area (mAU). A drastic increasing trend in the area of hemp was observed when the flow rate was increased. Second, when the wavelength was increased, the area was significantly reduced, whereas the mobile phase composition did not show any significant effect on the area of hemp.

As depicted in Figure 3a1−a3, the area of posaconazole has been assessed and the following changes have been described. As the flow rate increased, the area slightly increased. The most significant effect was seen in the case of wavelength: as the wavelength increased, the area of PCZ increased drastically. On the other hand, no change in the area was observed after changing the mobile phase composition.

https://doi.org/10.1021/acsomega.3c02097
ACS Omega 2023, 8, 30057−30067

Table 2. Results of Linearity for Calibration Curve Plots of Posaconazole

s. no	concentration of posaconazole (μg/mL)	average peak area ± SD[a] (n = 3) (mAU)	% RSD[b]
1	20	265,540 ± 1335.20	0.50
2	40	643,582.5 ± 3450.73	0.53
3	60	977,794 ± 53.53	0.005
4	80	1,309,046 ± 295.02	0.02
5	100	1,643,132 ± 19,772.82	1.19
6	120	1,909,879 ± 38,656.31	2.04

[a]Standard deviation. [b]Relative standard deviation.

Table 3. Results of Linearity for Calibration Curve Plots of Hemp Seed Oil

s. no	concentration of hemp seed oil (μg/mL)	average peak area ± SD[a] (n = 3) (mAU)	% RSD[b]
1	100	1788.33 ± 34.25	1.95
2	200	4441.66 ± 37.49	0.84
3	300	6234.66 ± 128.68	2.03
4	400	8554 ± 37.24	0.43
5	500	10,442.33 ± 128.53	1.22
6	600	12,159.33 ± 214.93	1.74

[a]Standard deviation. [b]Relative standard deviation.

In Figure 3b1−b3, the theoretical plates of posaconazole have been assessed and the following changes have been described. Theoretical plates were significantly increased initially and then decreased upon increasing the flow rate, wavelength, and composition.

In Figure 3c1−c3, the retention time of posaconazole has been assessed and the following changes have been described. When the composition of methanol increased, the retention time was significantly reduced, indicating the most significant change after changing the composition. On the other hand, no change in the RT was observed after changing the flow rate and wavelength.

The content of methanol influenced the area. The model F-value of 68,639.22 was observed for hemp oil, which implied that the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

Lastly, the software of designing indicated the value for an optimized result of factors such as the content of methanol (organic solvent), 1 mL/min rate of flow, and the wavelength along with other responses of these values were determined to be better than similar nanoparticles. The equations of the terms are given below (eqs 3−8)

$$\text{theoreticalplates of hemp oil: } 4311.76 - 406.00A + 8.08B + 0C + 9.25AB + 0AC + 0BC - 132.68A^2 + 68.45B^2 - 74.68C^2 \quad (3)$$

$$\text{retention time of hemp oil: } 2.91 - 0.0125A + 0.0133B - 0.1840C + 0AB + 0AC + 0BC + 0.0009A^2 - 0.0022B^2 + 0.0948C^2 \quad (4)$$

$$\text{area of hemp oil: } 7173.00 + 6010.17A - 1931.67B + 0C - 1413.0AB + 0AC + 0BC + 596.25A^2 - 796.75B^2 + 217.50C^2 \quad (5)$$

$$\text{theoretical plates of posaconazole: } 5075.46 + 3 + 50A - 3.00B + 0.2500C - 1.50AB - 0.50AC + 5.50BC - 11.55A^2 - 9.30B^2 - 6.93C^2 \quad (6)$$

$$\text{retention time of posaconazole: } 5.29 + 0A + 0.0036B - 0.8959C + 0AB + 0AC + 0.0108BC - 0A^2 + 0.0001B^2 + 0.2371C^2 \quad (7)$$

$$\text{area of posaconazole: } 1.245E + 06 + 10335.33A + 1.752E + 05B - 2987.75C + 0AB + 0AC + 0BC + 9440.53A^2 + 7673.91B^2 + 9211.91C^2 \quad (8)$$

The terms A, B, and C are independent variables, namely, flow rate (A), wavelength (nm) (B), and organic solvent content in the mobile phase or mobile phase composition (C), while the dependent variables are posaconazole retention time (RT) ($Y1$), hemp seed oil retention time ($Y4$), posaconazole area ($Y2$), hemp seed oil area ($Y5$), posaconazole theoretical plates (TP) ($Y3$), and hemp seed oil theoretical plates ($Y6$).

The wavelength selection for posaconazole hemp seed oil was very critical for effective separation of both analytes, and better separation was achieved at 240 nm wavelength because it was established by previous works that posaconazole was better separated at 260 nm and hemp seed oil showed better elution at much lower wavelength ranging from 214 to 228 nm. The C8 column showed better elution of both analytes at 240 nm with varying mobile phase compositions of methanol and water because of its composition, enabling low absorption of ionic compounds and good shape of peaks. Also, the C8 column performs better resolution at low pressure with a wider pH ranging from 2 to 9. Shim-pack Solar C18 and C8 columns

Table 4. Robustness of Posaconazole Observed at 235, 240, and 245 nm Wavelengths along with 25, 30, and 35 °C

number of formulations	peak area at 235 nm	peak area at 240 nm	peak area at 245 nm	peak area at 25 °C	peak area at 30 °C	peak area at 35 °C
F1	248,796	289,173	359,327	281,411	289,173	281,835
F2	245,594	287,919	358,356	280,341	287,919	280,770
F3	244,510	286,574	356,679	279,579	286,574	279,730
F4	244,657	286,666	353,810	275,421	286,666	279,239
F5	244,500	286,449	355,912	280,575	286,449	279,368
F6	244,525	286,499	356,354	281,132	286,499	279,539
mean	245,430.3	287,213.3	356,739.7	279,743.2	287,213.3	280,080.2
SD[a]	1702.04	1108.24	1935.31	2212.14	1108.24	1018.38
RSD[b]	0.69	0.38	0.54	0.79	0.38	0.36

[a]Standard deviation. [b]RSD: relative standard deviation, F: formulation.

https://doi.org/10.1021/acsomega.3c02097
ACS Omega 2023, 8, 30057−30067

Table 5. Robustness of Hemp Seed Oil Observed at 235, 240, and 245 nm wavelengths along with 25, 30, and 35° °C

number of formulations	peak area at 235 nm	peak area at 240 nm	peak area at 245 nm	peak area at 25 °C	peak area at 30 °C	peak area at 35 °C
F1	2025	1649	1110	2039	1649	198
F2	2026	1647	1101	2152	1647	191
F3	1992	1631	1099	2161	1631	225
F4	2017	1641	1094	2040	1641	243
F5	2000	1618	1085	2082	1618	200
F6	2018	1649	1118	2098	1649	209
mean	2013	1639.16	1101.16	2095.33	1639.16	211
SD[a]	13.88	12.43	11.65	52.80	12.43	19.56
RSD[b]	0.68	0.75	1.06	2.52	0.75	9.27

[a]Standard deviation. [b]RSD: relative standard deviation, F: formulation.

Table 6. Analytical Results of Accuracy of Posaconazole and Hemp Seed Oil

	concentration recovered (μg/mL)			% concentration found						
concentration (μg/mL)	R1[c]	R2	R3	R1	R2	R3	mean%	SD[a]	% RSD[b]	standard error
				Posaconazole						
20	20.26	19.95	20.11	101.3	99.8	100.6	100.5	0.775	0.771	0.45
40	40.31	39.86	39.28	100.8	99.7	98.2	99.5	1.291	1.297	0.75
60	60.19	59.51	60.32	100.3	99.2	100.5	100.0	0.725	0.725	0.42
				Hemp Seed Oil						
100	100.12	100.24	100.09	100.1	100.2	100.1	100.2	0.079	0.079	0.05
200	200.29	200.45	199.98	100.1	100.2	100.0	100.1	0.119	0.119	0.07
300	300.34	299.76	300.12	100.1	99.9	100.0	100.0	0.098	0.098	0.06

[a]Standard deviation. [b]Relative standard deviation. [c]Representative sample.

Table 7. Precision Table Showing Intraday and Interday Analysis of Posaconazole

	n1	n2	n3	avg	SD[a]	% RSD[b]
			Intraday			
20 (μg/mL)	266,562	264,879	264,518	265,320	1090.92	0.41
40 (μg/mL)	642,969	642,598	644,196	643,254	836.33	0.13
60 (μg/mL)	962,091	976,584	993,497	977,391	15,718.53	1.60
			Interday			
20 (μg/mL)	288,427	279,458	286,985	284,957	4816.25	1.69
40 (μg/mL)	696,176	694,158	694,125	694,820	1174.73	0.16
60 (μg/mL)	1,038,334	1,045,870	1,064,256	1,049,487	13,334.08	1.27

[a]Standard deviation. [b]Relative standard deviation.

Table 8. Precision Table Showing Intraday and Interday Analysis of Hemp Seed Oil

	n1	n2	n3	avg	SD[a]	% RSD[b]
			Intraday			
100	1750	1760	1725	1745	18.02	1.03
200	4218	4329	4298	4282	57.27	1.33
300	6135	6280	6289	6235	86.43	1.38
			Interday			
100	1592	1560	1550	1567	21.93	1.39
200	3994	3945	3897	3945	48.50	1.22
300	6585	6547	6500	6544	42.57	0.65

[a]Standard deviation. [b]Relative standard deviation.

are packed with high-purity silica gel or inertness. The silica gel has higher surface area and is fully end-capped with good hydrophobic retention, making this column very effective for analytes separations.

The optimization results of the developed method possessed the parameters such as theoretical plates, retention time, and area of both posaconazole and hemp oil using different factors and assessing their responses. By systematically varying these input parameters and analyzing the resulting output responses, we identified the optimal process conditions for producing posaconazole and hemp seed oil nanoparticles with their desired properties.

Method Validation. *System Suitability Test.* The mean retention time of posaconazole was observed to be 4.66 min and the % RSD of the area was determined to be 0.08. The mean retention time of hemp seed oil was estimated to be 2.77 min and the % RSD of the area evaluated was 1.81, as shown in Table 1. The injections of posaconazole had a % RSD of the column's theoretical plates of 0.08, and the tailing factor (TF) was determined to be 1.06. The injection of hemp seed oil produced 0.16% RSD of the column's theoretical plates, and the tailing factor was observed to be 1.22 as shown in Table 1. The theoretical plates had a larger number for posaconazole and hemp seed oil, which was deemed adequate for the suitability test of the system. The area had the % RSD within the recommended range of the standard. Therefore, the findings demonstrate that the suggested method of HPLC proved sufficient in producing data of adequate standard.

Specificity. The chromatograms of the exact content of the blank target solution of posaconazole and hemp seed oil

https://doi.org/10.1021/acsomega.3c02097
ACS Omega 2023, 8, 30057−30067

Table 9. Stabilities of Posaconazole and Hemp Seed Oil

		short term (% SD[d])			standard solution stability (% SD[d])	
level	thaw−freeze cycle (% SD[d])	4 h	12 h	long term (% SD) (−20 °C for 14 days)	room temperature at 6 h	−20 °C for 14 days
				Posaconazole		
LQC[a]	98.3 ± 2.7	99.3 ± 3.1	91.1 ± 2.9	99.2 ± 3.5		
MQC[b]	99.9 ± 3.3	99.7 ± 4	102.1 ± 2.1	101.5 ± 2.1	100.2 ± 2.7	100.5 ± 3.4
HQC[c]	98.5 ± 4.2	100.1 ± 3.2	99.3 ± 3.5	99.6 ± 3.4		
				Hemp Seed Oil		
LQC[a]	98.7 ± 3.5	98.2 ± 2.3	89.5 ± 4.5	100.4 ± 3.5		
MQC[b]	95.61 ± 2.3	99.1 ± 2.7	88.2 ± 3.2	99.6 ± 2.4	95.2 ± 2.3	99.3 ± 3.5
HQC[c]	97.7 ± 21.6	98.6 ± 3.2	86.9 ± 2.1	97.8 ± 5.2		

[a]Low-quality control formulation. [b]Moderate quality control formulation. [c]High-quality control formulation. [d]Standard deviation.

standard solution and their mixed solution were compared to assess specificity. 10 μL of the sample was injected into the HPLC system with the help of an autosampler. The retention times of posaconazole and hemp seed oil were found to be 4.6 ± 0.033 and 2.7 ± 0.074 min, respectively, as shown in Figure 4a. The retention times of both analytes were consistent in pure solutions, as shown in Figure 4b−d, and their mixtures. Furthermore, the coeluting peaks did not show any interference, thus indicating the specificity of the method.

Linearity. The curves of calibration illustrated linearity with posaconazole and hemp seed oil having their mean area of peak plotted over their respective concentrations on the graph. The findings show a linear correlation across the concentration of the analyte in the range of 20−120 and 100−600 μg/mL for posaconazole (Table 2) and hemp seed oil (Table 3), respectively. The equation for linearity and r^2 of posaconazole were $y = 16{,}502x - 30{,}331$ and $r^2 = 0.9978$ and those of hemp seed oil were $y = 20.622x + 52.422$ and $r^2 = 0.9957$, which were obtained from the data of regression. These findings suggested a relationship of linearity between the concentration of analytes and their mean area of resulting peaks.

Sensitivity. The LOD of posaconazole was 4.98 μg/mL and a LOQ of 15.08 μg/mL was observed. The LOD of hemp seed oil was 28.76 μg/mL and the LOQ of 90.17 μg/mL was observed. The findings reveal that the method is extremely sensitive compared to the earlier approaches.[35]

Robustness. The analytical method's robustness was assessed by examining the influence of minor changes in the column temperature, organic solvent content in the mobile phase, and the wavelength of detection. These findings revealed that minor changes in chromatographic conditions of the method did not produce a major effect on the retention time of analytes, theoretical plates, and area as shown in Tables 4 and 5 for posaconazole and hemp seed oil, respectively. These results suggest that the method is robust and reproducible.[36]

Accuracy. Accuracy is expressed as a percentage of recovery denoting the closeness to real value. The percentage recoveries of all of the quality control levels were 99.5−100.5% for posaconazole and 100.0−100.2% for hemp seed oil, as shown in Table 6. The findings were determined to be in the acceptable range, demonstrating the usability of an established approach for regular analysis of the drug. This method was ascertained to be accurate after a good recovery of values.[37]

Precision. Precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio of 158.897 indicated an adequate signal. For posaconazole, the area of the peak had a % RSD of 0.1−1.6 and was determined at all levels of quality control for interday precision, while hemp seed oil produced 0.6−1.3% RSD, respectively, as shown in Tables 7 and 8. It was observed that this method provides precise and reproducible results with 2% or lower % RSD, signifying the method's reliability and repeatability. This model can be used to navigate the design space.[38,39]

Stability Studies. The analyte's stability was studied at all levels of quality control in various storage conditions. As indicated in Table 9, the specificity of the projected concentration acquired after the three periods of thaw−freeze was observed to be at 37 °C for a short duration while it was −20 °C for a longer duration of 14 days in the ranges of 91−102.1% SD for posaconazole and 88.2−100.5% SD for hemp seed oil. The findings suggest that neither the drug nor the oil deteriorated over the various storage settings.[40−43]

The current literature has contradictory findings about the stability of posaconazole in acidic and alkaline mediums. This phenomenon can be addressed through fundamental molecular properties rather than through forced degradation studies as catalysts, residues of solvent, and impurities often change the synthesis process and alter the degradation pattern, notably in complex compounds with heavy molecular mass and more amounts of chemical groups such as posaconazole. As a result, health organizations implement rules and standards for stability and the critical need that chemicals for pharmaceutical formulations to be properly selected.[30,35,44]

CONCLUSIONS

The current study provides a unique approach for the assessment of posaconazole and hemp seed oil from nanomaterials, especially nanomicelles. Through the determination of appropriate organic solvent content in the mobile phase for effective separation of both the analytes, the experimental design enabled considerable advances in the efficiency and robustness of the method. In this method, we used an internal standard to standardize, quantify, and qualify the herbal moiety and created a reliable and inexpensive method with the help of HPLC, which makes it better than the existing analytical methods. The findings showed that using this analytical quality-by-design concept is cheap and adaptable to reducing the number of tests and producing more feasible information in a shorter duration. According to ICH criteria, the proposed real-time approach was rapid, uncomplicated, sensitive, efficient, and consistent. Furthermore, the new method demonstrated a significant level of applicability in nanoformulations for pharmacological analysis and simultaneous assessment of posaconazole and hemp seed oil as they produce a synergistic effect, which is a novel combination methodology

https://doi.org/10.1021/acsomega.3c02097
ACS Omega 2023, 8, 30057−30067

to treat IFD, which has not yet been reported. This combination provides immense advantages over the existing single posaconazole therapy significantly through augmentation of immunity in immunocompromised patients suffering from IFD. Eventually, it was determined that the suggested method of HPLC may be used to evaluate the pharmacokinetics and bioequivalence of posaconazole and hemp seed oil through which we can reduce the dose of the conventional synthetic posaconazole upto some extent and to increase the efficacy of the existing formulation using phytoadjuvant hemp oil laden with omega acids, which improve the existing condition of the sufferers. This novel formulation with a synergistic effect may prove to be the most effective therapy for IFIs in the pharmaceutical market.

AUTHOR INFORMATION

Corresponding Author

Yasmin Sultana − *Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi 110062, India;* orcid.org/0000-0002-3979-6574; Email: ysultana@jamiahamdard.ac.in

Authors

Anjali Rathee − *Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi 110062, India*

Pavitra Solanki − *Department of Pharmaceutics, Delhi Pharmaceutical Sciences and Research University, New Delhi 110017, India;* orcid.org/0000-0002-8032-9513

Surajpal Verma − *Department of Pharmaceutical Analysis, Delhi Pharmaceutical Sciences and Research University, New Delhi 110017, India*

Divya Vohora − *Department of Pharmacology, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi 110062, India*

Mohammad Javed Ansari − *Department of Pharmaceutics, College of Pharmacy, Prince Sattam Bin Abdul Aziz University, Al-kharj 11231, Saudi Arabia;* orcid.org/0000-0001-9266-7133

Alhussain Aodah − *Department of Pharmaceutics, College of Pharmacy, Prince Sattam Bin Abdul Aziz University, Al-kharj 11231, Saudi Arabia*

Kanchan Kohli − *Lloyd Institute of Management and Technology, Greater Noida, Uttar Pradesh 201306, India*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c02097

Author Contributions

Conceptualization and supervision: K.K. and Y.S.; methodology: A.R. and P.S.; software: S.V.; validation: A.R., S.V., and P.S.; formal analysis and drafting: D.V.; investigation, data curation, and writing—original draft preparation: A.R.; resources: Y.S.; writing—review and editing, and project administration: M.J.A.; visualization and funding acquisition: A.A. All authors have read and agreed to the published version of the manuscript." The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank DST-PURSE, Jamia Hamdard, New Delhi, for providing the financial assistance and necessary support required for conducting this research work.

REFERENCES

(1) Spellberg, B. Vaccines for Invasive Fungal Infections. *F1000 Med. Rep.* **2011**, *3*, No. 13.

(2) Stappers, M. H. T.; Brown, G. D. Host Immune Responses during Infections with *Candida Albicans*. In *Candida Albicans: Cellular and Molecular Biology*, 2nd ed.; 2017; pp 145−183.

(3) Garcia, C. V.; Costa, G. R.; Mendez, A. S. L. Stability-Indicating HPLC Method for Posaconazole Bulk Assay. *Sci. Pharm.* **2012**, *80*, 317−328.

(4) Lei, G.; He, D.; Liu, J.; Yan, W.; Gao, S.; Wang, L. Berberine and Itraconazole Are Not Synergistic in Vitro against Aspergillus Fumigatus Isolated from Clinical Patients. *Molecules* **2011**, *16*, 9218−9233.

(5) Panagopoulou, P.; Roilides, E. Evaluating Posaconazole, Its Pharmacology, Efficacy and Safety for the Prophylaxis and Treatment of Fungal Infections. *Expert Opin. Pharmacother.* **2022**, *23*, 175−199.

(6) Cornely, O. A.; Arikan-Akdagli, S.; Dannaoui, E.; Groll, A. H.; Lagrou, K.; Chakrabarti, A.; Lanternier, F.; Pagano, L.; Skiada, A.; Akova, M.; Arendrup, M. C.; Boekhout, T.; Chowdhary, A.; Cuenca-Estrella, M.; Freiberger, T.; Guinea, J.; Guarro, J.; de Hoog, S.; Hope, W.; Johnson, E.; Kathuria, S.; Lackner, M.; Lass-Flörl, C.; Lortholary, O.; Meis, J. F.; Meletiadis, J.; Muñoz, P.; Richardson, M.; Roilides, E.; Tortorano, A. M.; Ullmann, A. J.; van Diepeningen, A.; Verweij, P.; Petrikkos, G. ESCMID and ECMM Joint Clinical Guidelines for the Diagnosis and Management of Mucormycosis 2013. *Clin. Microbiol. Infect.* **2014**, *20*, 5−26.

(7) Cui, J.; Ren, B.; Tong, Y.; Dai, H.; Zhang, L. Synergistic Combinations of Antifungals and Anti-Virulence Agents to Fight against *Candida Albicans*. *Virulence* **2015**, *6*, 362−371.

(8) Chen, Y. L.; Lehman, V. N.; Averette, A. F.; Perfect, J. R.; Heitman, J. Posaconazole Exhibits In Vitro and In Vivo Synergistic Antifungal Activity with Caspofungin or FK506 against *Candida Albicans*. *PLoS One* **2013**, *8*, No. e57672.

(9) Russo, E. B.; Jiang, H. E.; Li, X.; Sutton, A.; Carboni, A.; Del Bianco, F.; Mandolino, G.; Potter, D. J.; Zhao, Y. X.; Bera, S.; Zhang, Y. B.; Lü, E. G.; Ferguson, D. K.; Hueber, F.; Zhao, L. C.; Liu, C. J.; Wang, Y. F.; Li, C. Sen. Phytochemical and Genetic Analyses of Ancient Cannabis from Central Asia. *J. Exp. Bot.* **2008**, *59*, 4171−4182.

(10) Appendino, G.; Gibbons, S.; Giana, A.; Pagani, A.; Grassi, G.; Stavri, M.; Smith, E.; Rahman, M. M. Antibacterial Cannabinoids from Cannabis Sativa: A Structure-Activity Study. *J. Nat. Prod.* **2008**, *71*, 1427−1430.

(11) Hernndez, L.; Palazon, J.; Navarro-Oca, A.The Pentacyclic Triterpenes-Amyrins: A Review of Sources and Biological Activities. In *Phytochemicals - A Global Perspective of Their Role in Nutrition and Health*, 2012; pp 487−502.

(12) Sabater-Jara, A. B.; Pedreño, M. A. Use of β-Cyclodextrins to Enhance Phytosterol Production in Cell Suspension Cultures of Carrot (*Daucus Carota* L.). *Plant Cell, Tissue Organ Cult.* **2013**, *114*, 249−258.

(13) VanDolah, H. J.; Bauer, B. A.; Mauck, K. F. Clinicians' Guide to Cannabidiol and Hemp Oils. *Mayo Clin. Proc.* **2019**, *94*, 1840−1851.

(14) Burton, R. A.; Andres, M.; Cole, M.; Cowley, J. M.; Augustin, M. A. Industrial Hemp Seed: From the Field to Value-Added Food Ingredients. *J. Cannabis Res.* **2022**, *4*, No. 45.

(15) Saini, D.; Fazil, M.; Ali, M. M.; Baboota, S.; Ameeduzzafar, A.; Ali, J. Formulation, Development and Optimization of Raloxifene-Loaded Chitosan Nanoparticles for Treatment of Osteoporosis. *Drug Delivery* **2015**, *22*, 823−836.

(16) Petrovici, A. R.; Simionescu, N.; Sandu, A. I.; Paraschiv, V.; Silion, M.; Pinteala, M. New Insights on Hemp Oil Enriched in

https://doi.org/10.1021/acsomega.3c02097
ACS Omega 2023, 8, 30057−30067

Cannabidiol: Decarboxylation, Antioxidant Properties and in Vitro Anticancer Effect. *Antioxidants* **2021**, *10*, 738.
(17) Kesharwani, S. S.; Kaur, S.; Tummala, H.; Sangamwar, A. T. Multifunctional Approaches Utilizing Polymeric Micelles to Circumvent Multidrug Resistant Tumors. *Colloids Surf., B* **2019**, *173*, 581−590.
(18) Cholkar, K.; Gunda, S.; Earla, R.; Pal, D.; Mitra, A. K. Nanomicellar Topical Aqueous Drop Formulation of Rapamycin for Back-of-the-Eye Delivery. *AAPS PharmSciTech* **2015**, *16*, 610−622.
(19) Chiappetta, D. A.; Hocht, C.; Taira, C.; Sosnik, A. Oral Pharmacokinetics of the Anti-HIV Efavirenz Encapsulated within Polymeric Micelles. *Biomaterials* **2011**, *32*, 2379−2387.
(20) Gong, J.; Chen, M.; Zheng, Y.; Wang, S.; Wang, Y. Polymeric Micelles Drug Delivery System in Oncology. *J. Controlled Release* **2012**, *159*, 312−323.
(21) Wu, X. L.; Kim, J. H.; Koo, H.; Bae, S. M.; Shin, H.; Kim, M. S.; Lee, B. H.; Park, R. W.; Kim, I. S.; Choi, K.; Kwon, I. C.; Kim, K.; Lee, D. S. Tumor-Targeting Peptide Conjugated PH-Responsive Micelles as a Potential Drug Carrier for Cancer Therapy. *Bioconjugate Chem.* **2010**, *21*, 208−213.
(22) Javadzadeh, Y.; Azharshekoufeh Bahari, L. Therapeutic Nanostructures for Dermal and Transdermal Drug Delivery. *Nano-Microscale Drug Delivery Syst.* **2017**, 131−146.
(23) Ahmad Khan, M. S.; Ahmad, I. Herbal Medicine: Current Trends and Future Prospects. *New Look Phytomed.* **2018**, 3−13.
(24) Sharma, K.; Guleria, S.; Razdan, V. K.; Babu, V. Synergistic Antioxidant and Antimicrobial Activities of Essential Oils of Some Selected Medicinal Plants in Combination and with Synthetic Compounds. *Ind. Crops Prod.* **2020**, *154*, No. 112569.
(25) Herman, A.; Herman, A. P. Herbal Products and Their Active Constituents Used Alone and in Combination with Antifungal Drugs against Drug-Resistant Candida Sp. *Antibiotics* **2021**, *10*, 655.
(26) Gong, M.; Wei, W.; Hu, Y.; Jin, Q.; Wang, X. Structure Determination of Conjugated Linoleic and Linolenic Acids. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2020**, *1153*, No. 122292.
(27) Song, Z.; Li, S.; Guan, Y.; Wang, S.; Wang, Y.; Yang, G.; Zhang, X.; Li, J.; Song, W.; Zhou, C.; Chen, L. Facile Synthesis of Zirconia-Coated Mesoporous Silica Particles by Hydrothermal Strategy under Low Potential of Hydrogen Conditions and Functionalization with Dodecylphosphonic Acid for High-Performance Liquid Chromatography. *J. Chromatogr. A* **2020**, *1612*, No. 460659.
(28) Dejaegher, B.; Heyden, Y. V. Ruggedness and Robustness Testing. *J. Chromatogr. A* **2007**, *1158*, 138−157.
(29) Magdy, G.; Abdel Hakiem, A. F.; Belal, F.; Abdel-Megied, A. M. A Novel Quality by Design Approach for Development and Validation of a Green Reversed-Phase HPLC Method with Fluorescence Detection for the Simultaneous Determination of Lesinurad, Febuxostat, and Diflunisal: Application to Human Plasma. *J. Sep. Sci.* **2021**, *44*, 2177−2188.
(30) Solanki, P.; Ansari, M. D.; Alam, M. I.; Aqil, M.; Ahmad, F. J.; Sultana, Y. *Precision Engineering Designed Phospholipid-Tagged Pamidronate Complex Functionalized SNEDDS for the Treatment of Postmenopausal Osteoporosis*; Springer: US, 2022; pp 883−913.
(31) Derakhshandeh, K.; Soheili, M.; Dadashzadeh, S.; Saghiri, R. Preparation and in Vitro Characterization of 9-Nitrocamptothecin-Loaded Long Circulating Nanoparticles for Delivery in Cancer Patients. *Int. J. Nanomed.* **2010**, *5*, 463−471.
(32) Solanki, P. Recent Innovations in Oromucosal Drug Delivery. *Global J. Pharm. Res.* **2013**, *2*, 1813−1821.
(33) Solanki, P.; Sultana, Y.; Singh, S. Traditional Medicine: Exploring Their Potential in Overcoming Multi-Drug Resistance. *Strategies Overcome Superbug Invasion Emerging Res. Oppor.* **2021**, 118−129.
(34) Pankaj, M.; Pavitra, K.; Mangla, B.; Aggarwal, G. Formulation Development, Optimization by Box - Behnken Design, and In Vitro Characterization of Gefitinib Phospholipid Complex Based Nanoemulsion Drug Delivery System. *J. Pharm. Innovation* **2022**, 1−13.
(35) Tiwari, R.; Kumar, A.; Solanki, P.; Dhobi, M.; Sundaresan, V.; Kalaiselvan, V.; Raghuvanshi, R. S. Analytical Quality-by-Design (AQbD) Guided Development of a Robust HPLC Method for the Quantification of Plumbagin from Plumbago Species. *J. Liq. Chromatogr. Relat. Technol.* **2021**, *44*, 529−537.
(36) Ganorkar, S. B.; Dhumal, D. M.; Shirkhedkar, A. A. Development and Validation of Simple RP-HPLC-PDA Analytical Protocol for Zileuton Assisted with Design of Experiments for Robustness Determination. *Arabian J. Chem.* **2017**, *10*, 273−282.
(37) Legay, F.; Gauron, S.; Deckert, F.; Gosset, G.; Pfaar, U.; Ravera, C.; Wiegand, H.; Schran, H. Development and Validation of a Highly Sensitive RIA for Zoledronic Acid, a New Potent Heterocyclic Bisphosphonate, in Human Serum, Plasma and Urine. *J. Pharm. Biomed. Anal.* **2002**, *30*, 897−911.
(38) Kumar, R.; Solanki, P.; Chandra, A.; Modi, K. N. Medicated Chewing Gum-A Novel Drug Delivery System: An Updated Review. *Am. J. Adv. Drug Delivery* **2014**, *2*, 434−450.
(39) Solanki, P.; Ansari, M. D.; Anjali; Khan, I.; Jahan, R. N.; Nikita; Pandit, J.; Aqil, M.; Ahmad, F. J.; Sultana, Y. Repurposing Pentosan Polysulfate Sodium as Hyaluronic Acid Linked Polyion Complex Nanoparticles for the Management of Osteoarthritis: A Potential Approach. *Med. Hypotheses* **2021**, *157*, No. 110713.
(40) Solanki, P.; Kumar Patel, P.; Garg, B. Formulation Development and Evaluation of Mucoadhesive Buccal Patches of Metoprolol Tartrate. *Int. J. Biol. Pharm. Res.* **2013**, *4*, 1108−1122.
(41) Khan, I.; Pandit, J.; Ahmed, S.; Zameer, S.; Nikita; Ahmad, S.; Bano, S.; Ansari, M. D.; Solanki, P.; Jahan, R. N.; Waheed, A.; Aqil, M.; Sultana, Y. Development and Evaluation of Biodegradable Polymeric Lomustine Nanofibres for the Efficient Tumor Targeting: In Vitro Characterization, Ex Vivo Permeation and Degradation Study. *J. Drug Delivery Sci. Technol.* **2022**, *75*, No. 103685.
(42) Ansari, M. D.; Saifi, Z.; Pandit, J.; Khan, I.; Solanki, P.; Sultana, Y.; Aqil, M. Spanlastics a Novel Nanovesicular Carrier: Its Potential Application and Emerging Trends in Therapeutic Delivery. *AAPS PharmSciTech* **2022**, *23*, No. 112.
(43) Dutta, M.; Solanki, P.; Chaudhary, G.; Sahoo, P. K.; Dutt, R. Development And Validation Of Analytical Method For The Simultaneous Estimation Of Imiquimod And Thymoquinone. *Eur. Chem. Bull.* **2023**, 826−835.
(44) Moraes do Carmo, A. C.; Pereira, R. S.; Gratieri, T. Brazilian Requirements for Stability Indicating Methods. *TrAC, Trends Anal. Chem.* **2018**, *98*, 58−63.

https://doi.org/10.1021/acsomega.3c02097
ACS Omega 2023, 8, 30057−30067