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Development, Box-Behnken Optimization, and Evaluation of Itraconazole Nanocrystal-Loaded Bioadhesive Nail Lacquer for Enhanced Transungual Delivery and Improved Antifungal Activity against *Trichophyton rubrum*

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ABSTRACT: The present study aimed to develop and optimize an itraconazole nanocrystal-loaded bioadhesive nail lacquer for improved transungual delivery. Itraconazole nanocrystals were prepared by wet media milling using Poloxamer 407 as stabilizer. A three-factor, three-level Box-Behnken design was used to study the effects of Poloxamer 407 concentration, milling speed, and milling time on particle size, polydispersity index, and apparent saturation solubility. The optimized nanocrystals showed a particle size of 192.1 ± 3.4 nm, PDI of 0.202 ± 0.009 , zeta potential of -21.8 ± 1.6 mV, and apparent saturation solubility of 21.1 ± 0.6 µg/mL. Nanocrystallisation markedly improved itraconazole dissolution. The optimized nanocrystals were incorporated into an HPMC E15-based bioadhesive nail lacquer containing PEG 400 and thioglycolic acid. The formulation showed acceptable drug content, drying time, viscosity, film properties, and adhesion. Ex vivo permeation studies showed a steady-state flux of 1.48 ± 0.09 µg/cm²/h and nail drug retention of 286.7 ± 17.5 µg/g. The optimized formulation produced 2.39-fold higher flux than conventional itraconazole lacquer. Confocal microscopy and SEM supported enhanced nail penetration. Antifungal activity against *Trichophyton rubrum* was also improved. The formulation remained stable during

3 months of storage. These findings support nanocrystal-loaded bioadhesive nail lacquer as a promising approach for localized itraconazole delivery in onychomycosis.

1. INTRODUCTION

Onychomycosis is a fungal infection of the nail plate and nail bed. It is commonly caused by dermatophytes, especially *Trichophyton rubrum*. The disease may produce nail thickening, discoloration, brittleness, and separation of the nail plate. Treatment is often difficult because the nail acts as a strong barrier to drug penetration. Itraconazole is a broad-spectrum triazole antifungal drug. It is effective against dermatophytes, yeasts, and several other fungi. However, itraconazole has very poor aqueous solubility. This limits its dissolution and local availability when used in topical formulations. Systemic itraconazole is effective, but prolonged oral therapy may produce drug interactions and systemic adverse effects. A localized transungual delivery system may reduce systemic exposure and provide higher drug levels at the infected nail (Al-Obaidi et al., 2022; Bellmann et al., 2022; Kravvas et al., 2018; Nirbhavane et al., 2020).

The human nail plate is highly keratinized. Its compact structure and disulfide-rich keratin network restrict drug diffusion. Hydrophobic and poorly soluble drugs such as itraconazole face an additional barrier because sufficient dissolved drug must be available before diffusion can occur. Therefore, an effective topical system should improve both drug dissolution and nail penetration. Drug nanocrystals can help address the poor solubility of itraconazole. Nanocrystals consist mainly of pure drug particles in the nanometre range and require only a small amount of stabilizer. Reduction in particle size increases surface area and may improve apparent saturation solubility and dissolution rate. Wet media milling is a suitable method for preparing nanocrystals of poorly soluble drugs. Process variables such as stabilizer concentration, milling speed, and milling time can strongly affect nanocrystal size and uniformity. These variables therefore require systematic optimization (Bellmann et al., 2022; Gupta et al., 2025; Joly-Tonetti et al., 2024; Kishishita et al., 2024; Scandorieiro et al., 2024; Valido et al., 2025; Watjer et al., 2024).

Quality by Design tools can be used to study such formulation and process variables efficiently. The Box-Behnken design is useful for evaluating three independent factors at three levels with fewer experimental runs than a full factorial design. It also allows estimation of interaction and quadratic effects. In the present study, Poloxamer 407 concentration, milling speed, and milling time were selected as independent variables. Particle size, PDI, and apparent saturation solubility were selected as critical responses. For effective transungual delivery, the optimized nanocrystals were further incorporated into a bioadhesive nail lacquer. HPMC E15 was selected as the film-forming polymer. PEG 400 was used as the plasticizer. Thioglycolic acid was included as a nail permeation enhancer because it can alter the keratin structure and improve diffusion through the nail plate (Elabbasi et al., 2024; Goyal et al., 2024; Joly-Tonetti et al., 2024; Kishishita et al., 2024).

The aim of the present study was to develop and optimize itraconazole nanocrystals and incorporate them into a bioadhesive nail lacquer for improved transungual delivery. The optimized system was evaluated for physicochemical properties, dissolution, ex vivo permeation, nail retention, confocal penetration, nail-surface morphology, antifungal activity, and short-term stability.

2. MATERIALS AND METHODS

2.1 Materials

Itraconazole was used as the model antifungal drug and received as a gift sample from Zoron pharma, Baddi, Himachal Pradesh. Poloxamer 407 was used as the stabilizer during nanocrystal preparation. Hydroxypropyl methylcellulose E15 was used as the film-forming and bioadhesive polymer. Polyethylene glycol 400 was used as the plasticizer. Thioglycolic acid was used as the transungual permeation enhancer. Ethanol and purified water were used as formulation solvents. Potassium dihydrogen phosphate, disodium hydrogen phosphate, methanol, acetonitrile, and other analytical-grade reagents were used during drug estimation and permeation studies. All solvents used for chromatographic analysis were of HPLC grade. Purified water was used throughout the experimental work. Human nail clippings were used for ex vivo permeation and nail-retention studies. Healthy nail samples without visible fungal infection, discoloration, or structural damage were selected. The samples were cleaned before use and stored under suitable conditions until further experiments.

2.2 Analytical Method for Itraconazole

Itraconazole was quantified using a validated high-performance liquid chromatography method. A reverse-phase C18 column was used for separation. The mobile phase consisted of acetonitrile and aqueous buffer in a suitable ratio. The mobile phase composition was adjusted during preliminary trials to obtain a sharp and symmetrical itraconazole peak. The flow rate was maintained at approximately 1.0 mL/min. Detection was performed using a UV detector at a wavelength suitable for itraconazole analysis. A fixed injection volume was used for all samples. A stock solution of itraconazole was prepared in methanol. Working standard solutions were prepared by serial dilution. A calibration curve was constructed by plotting peak area against itraconazole concentration. The method was evaluated for linearity, precision, accuracy, and repeatability before its use for formulation analysis (Choi et al., 2023; Frasci-Nieto et al., 2026; Manchuri et al., 2024).

2.3 Preliminary Screening of Nanocrystal Processing Conditions

Preliminary experiments were carried out before Box-Behnken optimisation. These trials were performed to identify a practical range for stabilizer concentration, milling speed, and milling time. Itraconazole concentration was maintained at 2% w/v during nanocrystal preparation. Poloxamer 407 was screened as the stabilizer because of its ability to provide steric stabilization. Milling conditions were adjusted to obtain a nanosized dispersion with low polydispersity and without visible sedimentation. The ranges selected from preliminary studies were used as the factor levels in the Box-Behnken design (Alsadi et al., 2022; Sengel-Turk et al., 2022; Singh et al., 2022; Tamilarasan et al., 2022; Yu & He, 2017).

2.4 Preparation of Itraconazole Nanocrystals

Itraconazole nanocrystals were prepared by wet media milling. Itraconazole was dispersed in an aqueous solution of Poloxamer 407. The drug concentration was kept constant at 2% w/v. Yttria-stabilized zirconia beads with a diameter of approximately 0.1-0.2 mm were used as the milling medium. The drug suspension and milling beads were transferred to the milling vessel. Milling was performed at the speed and duration specified by the experimental design. The temperature of the dispersion was controlled during processing. It was maintained below 30°C to reduce the possibility of thermal degradation or undesirable solid-state changes. After milling, the dispersion was separated from the milling beads. The resulting nanocrystal dispersion was collected and used for particle-size analysis, polydispersity measurement, solubility testing, and further optimisation studies (Bonhoeffer et al., 2022; Cristina Cardia et al., 2022; Kalhapure et al., 2022; Ma et al., 2022).

2.5 Box-Behnken Experimental Design

A three-factor, three-level Box-Behnken design was used to optimize the preparation of itraconazole nanocrystals. Design-Expert software was used for experimental design and statistical analysis. Three independent variables were selected. The concentration of Poloxamer 407 was selected as factor X1. Milling speed was selected as factor X2. Milling time was selected as factor X3. The selected factor levels were as follows (Alsadi et al., 2022; Gares et al., 2023; Shrivastava & Daharwal, 2022; Yu & He, 2017):

Table 1. Selected factor levels

Factor	Variable	Low level (-1)	Centre level (0)	High level (+1)
X1	Poloxamer 407 concentration (% w/v)	0.25	0.50	0.75
X2	Milling speed (rpm)	800	1200	1600
X3	Milling time (h)	6	12	18

A total of 17 experimental runs were generated. Five centre-point runs were included to estimate experimental variability. Three dependent responses were evaluated. Mean particle size was selected as response Y1. Polydispersity index was selected as response Y2. Apparent saturation solubility was selected as response Y3. Particle size and PDI were minimized during numerical

optimization. Apparent saturation solubility was maximized. The experimental data were fitted to suitable mathematical models. Linear, two-factor interaction, and quadratic models were examined. The final model was selected using analysis of variance, model significance, lack of fit, coefficient of determination, adjusted coefficient of determination, predicted coefficient of determination, coefficient of variation, and adequate precision (Alsadi et al., 2022; Gares et al., 2023; Shrivastava & Daharwal, 2022; Yu & He, 2017).

2.6 Measurement of Particle Size and Polydispersity Index

The mean particle size and PDI of each experimental formulation were measured by dynamic light scattering. An appropriate quantity of nanocrystal dispersion was diluted with filtered purified water before analysis. Dilution was performed carefully to avoid multiple scattering effects. Measurements were performed at controlled temperature. Each formulation was analysed in triplicate. Particle size was reported in nanometres. PDI was used to describe the width of the particle-size distribution. A lower PDI value was considered desirable because it represented a more uniform nanocrystal population (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.7 Zeta Potential

Zeta potential was measured for the optimized nanocrystal formulation. The nanocrystal dispersion was suitably diluted before analysis. The electrophoretic mobility of the dispersed particles was measured using a zeta-potential analyser. The result was expressed in millivolts.

The measurement was used to support the assessment of physical stability. The stabilizing contribution of Poloxamer 407 was also considered because it provides steric stabilization in addition to electrostatic effects (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.8 Apparent Saturation Solubility

The apparent saturation solubility of coarse itraconazole and itraconazole nanocrystals was determined using the shake-flask method. An excess quantity of the drug or nanocrystal formulation was added to a fixed volume of dissolution medium. The samples were incubated under continuous agitation for sufficient time to approach equilibrium. The resulting dispersions were separated using a suitable centrifugation and filtration procedure. The clear supernatant was analysed for itraconazole content using the validated HPLC method. The result was expressed as micrograms of itraconazole per millilitre. The apparent solubility of optimized nanocrystals was compared with that of untreated itraconazole (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.9 Numerical Optimisation and Model Validation

Numerical optimisation was performed using the desirability function. The optimisation goals were set as follows

- particle size: minimize;
- PDI: minimize;
- apparent saturation solubility: maximize.

The formulation showing the highest overall desirability was selected as the optimized nanocrystal formulation. An independent optimized batch was then prepared using the predicted factor settings. The experimentally observed responses were compared with the predicted responses. Prediction error was calculated using the following equation (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024):

$$\text{Prediction error (\%)} = [(\text{Observed value} - \text{Predicted value}) / \text{Predicted value}] \times 100$$

A small prediction error was considered evidence of adequate model validity.

2.10 Morphological Examination

The morphology of optimized itraconazole nanocrystals was examined using scanning electron microscopy. The nanocrystal sample was dried before analysis. A small amount of sample was mounted on the specimen holder. The sample was coated with

a thin conductive layer when required. The surface structure, particle shape, and degree of aggregation were examined at suitable magnifications (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.11 Fourier Transform Infrared Spectroscopy

FTIR spectroscopy was used to examine possible interactions between itraconazole and formulation components. Spectra were recorded for pure itraconazole, Poloxamer 407, and optimized itraconazole nanocrystals. Samples were scanned over the standard infrared spectral range. The principal characteristic peaks of itraconazole were identified. Changes in peak position, intensity, or shape were examined to assess possible drug-exipient interactions (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.12 Differential Scanning Calorimetry

Differential scanning calorimetry was performed to investigate the thermal behaviour of itraconazole before and after nanocrystal preparation. Samples of pure itraconazole and optimized nanocrystals were placed in aluminium pans. The samples were heated at a controlled rate under an inert atmosphere. The thermograms were examined for the melting endotherm of itraconazole. Changes in peak intensity, peak shape, and transition temperature were used to assess changes in crystallinity (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.13 Powder X-Ray Diffraction

PXRD analysis was carried out to examine the crystalline state of itraconazole. Diffractograms were recorded for pure drug and optimized nanocrystals over an appropriate diffraction-angle range. Characteristic crystalline peaks of itraconazole were compared between the samples. Reduction in peak intensity or changes in diffraction patterns were used to evaluate possible changes in crystal order after wet milling (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.14 In Vitro Dissolution Study

The dissolution behaviour of coarse itraconazole and optimized itraconazole nanocrystals was compared. An amount equivalent to a fixed dose of itraconazole was placed in the dissolution medium. The medium contained a suitable surfactant to maintain sink conditions for the poorly soluble drug. The temperature was maintained at $37 \pm 0.5^\circ\text{C}$. Samples were collected at predetermined time intervals. An equal volume of fresh dissolution medium was added after each withdrawal. The samples were filtered and analysed by HPLC. The cumulative percentage of itraconazole dissolved was plotted against time (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.15 Preparation of Itraconazole Nanocrystal-Loaded Bioadhesive Nail Lacquer

The optimized itraconazole nanocrystals were incorporated into a hydroalcoholic bioadhesive nail-lacquer system. The target itraconazole concentration in the final formulation was 1% w/v. The final composition per 10 mL was designed as follows (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024):

Table 2. Final composition per 10 mL of the formulation

Component	Quantity	Function
Itraconazole nanocrystals	Equivalent to 100 mg ITZ	Antifungal drug
HPMC E15	400 mg	Film-forming and bioadhesive polymer
PEG 400	100 mg	Plasticizer
Thioglycolic acid	250 μL	Nail permeation enhancer
Ethanol	6 mL	Volatile solvent
Purified water	q.s. to 10 mL	Vehicle

HPMC E15 was gradually dispersed in the hydroalcoholic vehicle under continuous stirring. PEG 400 was then added as the plasticizer. Thioglycolic acid was incorporated slowly with continuous mixing. The optimized itraconazole nanocrystal dispersion was then added. The formulation was mixed until a uniform lacquer was obtained. The final volume was adjusted with purified water. The formulation was stored in a tightly closed container to reduce solvent evaporation.

2.16 Physicochemical Evaluation of the Nail Lacquer

The optimized nail lacquer was evaluated for appearance, colour, homogeneity, pH, viscosity, drug content, and physical stability. The formulation was visually examined for phase separation, crystal formation, sedimentation, and precipitation. Viscosity was measured using a rotational viscometer at controlled temperature. For drug-content estimation, a known amount of the lacquer was diluted with a suitable solvent. Itraconazole concentration was then determined by HPLC (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.17 Drying Time and Film Characteristics

A fixed quantity of the lacquer was applied uniformly to a clean glass surface. The time required to form a dry and non-sticky film was recorded. The dried film was examined for smoothness, continuity, transparency, brittleness, cracking, and ease of removal. Film flexibility was evaluated by repeated bending or folding. A film that remained intact without visible cracking was considered flexible. Non-volatile content was determined by weighing a known amount of lacquer before and after complete evaporation of volatile components (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.18 Bioadhesion Study

The bioadhesive performance of the optimized lacquer was evaluated using a texture analyser.

Human nail samples were used as the biological substrate. A defined quantity of lacquer was applied to the nail surface and allowed to form a film. The coated nail surface was brought into controlled contact with the opposing probe. The force required for separation was recorded. Peak adhesive force and work of adhesion were calculated. Measurements were performed in triplicate (Dehari et al., 2022; Monti et al., 2022; Puri et al., 2022; Scandorieiro et al., 2024).

2.19 Ex Vivo Transungual Permeation Study

Ex vivo permeation was studied using Franz diffusion cells. Healthy human nail clippings were hydrated before use (Dehari et al., 2022). Each nail sample was mounted between the donor and receptor compartments. The dorsal surface of the nail faced the donor compartment. The receptor compartment was filled with a hydroalcoholic phosphate-buffered medium capable of maintaining sink conditions for itraconazole. The receptor phase was maintained at $37 \pm 0.5^\circ\text{C}$ and stirred continuously. A fixed dose of formulation was applied to the exposed nail surface. Samples were withdrawn from the receptor compartment at 2, 4, 6, 8, 12, 24, 36, 48, and 72 h. The same volume of fresh receptor medium was added after each sampling. Itraconazole concentration was determined using HPLC. The cumulative amount of itraconazole permeated per unit area was plotted against time. Steady-state flux was calculated from the slope of the linear region of the permeation profile (Dehari et al., 2022).

$$J_{ss} = dQ / (A \times dt)$$

where (C₀) is the initial drug concentration in the donor formulation.

2.20 Drug Retention in the Nail Plate

At the end of the permeation study, the nail samples were removed from the Franz diffusion cells. The nail surface was carefully cleaned to remove residual formulation. The samples were dried and weighed. Each nail sample was cut into small pieces. Itraconazole was extracted using a suitable organic solvent with vortex mixing and sonication. The extraction medium was centrifuged and filtered. The itraconazole content was quantified using HPLC. Drug retention was expressed as micrograms of itraconazole per gram of nail tissue (Dehari et al., 2022).

2.21 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy was used to examine the depth of penetration through the nail plate. A fluorescent marker formulation was prepared as a visual surrogate for the optimized delivery system. Rhodamine B was used as the fluorescent probe. The fluorescent formulation was applied to the nail surface under conditions similar to those used during permeation

testing. After treatment, the nail was washed and sectioned. Cross-sectional images were obtained using confocal microscopy. The depth and intensity of fluorescence within the nail were compared with those obtained from the control formulation (Dehari et al., 2022).

2.22 Scanning Electron Microscopy of Treated Nail

SEM was used to study surface changes in the nail after treatment. Three sample groups were examined. These included untreated nail, nail treated with conventional itraconazole formulation, and nail treated with the optimized thioglycolic-acid-containing nanocrystal lacquer. The nail samples were dried and prepared for SEM examination. Changes in surface morphology, roughness, keratin arrangement, and visible structural opening were compared between the groups (Dehari et al., 2022).

2.23 Antifungal Activity

The antifungal activity of the optimized itraconazole nanocrystal-loaded nail lacquer was evaluated against *Trichophyton rubrum*. A standardized fungal inoculum was prepared under aseptic conditions. The antifungal effects of blank lacquer, conventional itraconazole formulation, itraconazole nanocrystals, and optimized ITZ nanocrystal-loaded bioadhesive lacquer were compared. Agar diffusion was used as the initial screening method. The diameter of the inhibition zone was measured after incubation. Minimum inhibitory concentration was also determined using a broth-dilution procedure. The lowest itraconazole concentration that prevented visible fungal growth was recorded as the MIC (Ahmed et al., 2022; Al-Obaidi et al., 2022; El-Zahar et al., 2022; Mi et al., 2022; Omar et al., 2021).

2.24 Stability Study

The optimized nail lacquer was subjected to short-term stability testing. Samples were stored under the following conditions: $25 \pm 2^\circ\text{C}/60 \pm 5\%$ relative humidity and $40 \pm 2^\circ\text{C}/75 \pm 5\%$ relative humidity. The study was continued for 3 months. Samples were examined initially and after 1, 2, and 3 months. The formulation was evaluated for appearance, precipitation, particle size, PDI, viscosity, drug content, and drying time. Any major change in these parameters was considered evidence of physical or chemical instability (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human, 2003; Organisation for Economic & Development, 1981).

2.25 Statistical Analysis

All quantitative experiments were performed in triplicate unless otherwise stated. Results were expressed as mean $\pm$ standard deviation. Box-Behnken data were analysed using Design-Expert software. Analysis of variance was used to determine the significance of the experimental models and individual model terms. For comparative formulation studies, one-way analysis of variance followed by Tukey's multiple-comparison test was used. A value of $p < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Preliminary Formulation Development

Preliminary trials showed that wet media milling could reduce the particle size of itraconazole effectively. Formulations prepared without sufficient Poloxamer 407 showed rapid aggregation after milling. Increasing the stabilizer concentration improved dispersion stability and reduced visible particle aggregation. Low milling speeds produced relatively large particles. Prolonged milling at higher speed produced a finer and more uniform dispersion. However, excessive stabilizer concentration or prolonged processing produced only limited additional reduction in particle size. These observations supported the selected experimental ranges of 0.25-0.75% w/v Poloxamer 407, 800-1600 rpm milling speed, and 6-18 h milling time. The selected ranges were therefore used in the Box-Behnken design.

3.2 Box-Behnken Optimisation of Itraconazole Nanocrystals

A total of 17 experimental runs were evaluated. The observed particle size ranged from 198.0 to 289.0 nm. PDI ranged from 0.211 to 0.332. Apparent saturation solubility ranged from 8.4 to 21.9 $\mu\text{g/mL}$.

Table 3. Box-Behnken experimental runs and observed responses

Run	X1: P407	X2: Speed	X3: Time	Particle size (nm)	PDI	Apparent solubility (µg/mL)
1	-1	-1	0	288.0	0.332	9.5
2	+1	-1	0	235.0	0.259	15.0
3	-1	+1	0	227.0	0.250	18.2
4	+1	+1	0	204.0	0.217	20.7
5	-1	0	-1	277.0	0.317	9.8
6	+1	0	-1	237.0	0.259	15.7
7	-1	0	+1	228.0	0.250	17.3
8	+1	0	+1	202.0	0.214	21.2
9	0	-1	-1	289.0	0.332	8.4
10	0	+1	-1	232.0	0.254	16.4
11	0	-1	+1	237.0	0.261	15.7
12	0	+1	+1	198.0	0.211	21.9
13	0	0	0	205.5	0.219	18.8
14	0	0	0	204.0	0.217	18.5
15	0	0	0	206.2	0.220	18.7
16	0	0	0	204.3	0.216	18.4
17	0	0	0	205.3	0.218	18.6

The relatively small variation between the five centre-point batches showed acceptable process repeatability. Quadratic models provided the best fit for all three responses.

3.3 Statistical Analysis of the Box-Behnken Models

The quadratic models were highly significant. High R^2 values were obtained for particle size, PDI, and apparent saturation solubility.

Table 4. Statistical parameters of the fitted response models

Parameter	Particle size	PDI	Apparent solubility
Model	Quadratic	Quadratic	Quadratic
R^2	0.9988	0.9989	0.9980
Adjusted R^2	0.9972	0.9974	0.9954
Predicted R^2	0.9834	0.9874	0.9736
CV (%)	0.71	0.84	1.63
Model p-value	<0.0001	<0.0001	<0.0001

The difference between adjusted R^2 and predicted R^2 was small for all responses. This indicated good agreement between model fitting and predictive performance. The low CV values also indicated acceptable experimental precision.

The coded quadratic equations were:

Particle size:

PDI:

$$Y_1 = 205.06 - 17.75X_1 - 23.50X_2 - 21.25X_3 + 7.50X_1X_2 + 3.50X_1X_3 + 4.50X_2X_3 + 15.22X_1^2 + 18.22X_2^2 + 15.72X_3^2$$

Apparent saturation solubility:

$$Y_3 = 18.60 + 2.225X_1 + 3.575X_2 + 3.225X_3 - 0.750X_1X_2 - 0.500X_1X_3 - 0.450X_2X_3 - 1.175X_1^2 - 1.575X_2^2 - 1.425X_3^2$$

The coefficients showed that milling speed had the strongest effect on particle size and PDI. Milling time also produced a marked effect. Poloxamer 407 concentration showed a smaller but significant contribution.

3.4 Effect of Formulation Variables on Particle Size

Particle size decreased as the concentration of Poloxamer 407 increased from the lower level toward the intermediate-to-high region. At low stabilizer concentration, the newly generated surfaces were insufficiently protected. This favoured particle aggregation. Increasing Poloxamer 407 improved surface coverage and steric stabilization. Milling speed produced a strong negative effect on particle size. Increasing the speed increased collision frequency between the drug crystals and milling beads. This increased mechanical energy input and promoted crystal fragmentation. Milling time showed a similar effect. Longer milling allowed repeated fracture of the itraconazole crystals. However, the positive quadratic terms suggested that the benefit became smaller near the upper processing levels. The smallest particle size among the experimental batches was 198.0 nm, observed at the combination of centre-level stabilizer, high milling speed, and high milling time.

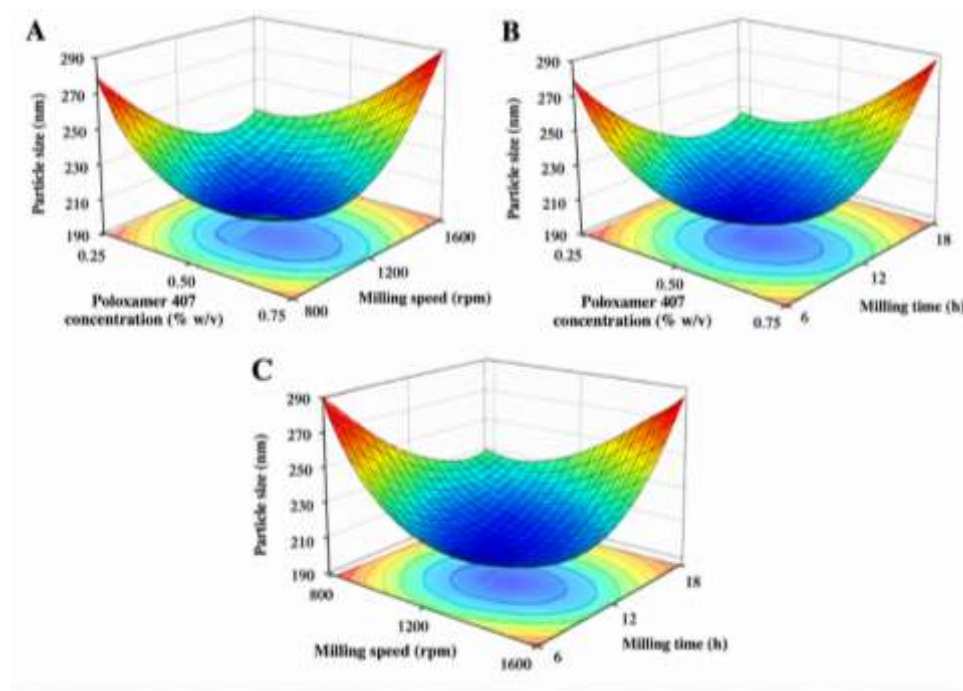


Figure 1. Three-dimensional response-surface plots showing the combined effects of Poloxamer 407 concentration, milling speed, and milling time on the particle size of itraconazole nanocrystals.

3.5 Effect of Variables on PDI

PDI followed a trend similar to particle size. The highest PDI was 0.332, while the lowest value was 0.211. The lower values obtained at increased milling speed and milling time indicated the formation of a more uniform particle population. High PDI

at low milling intensity was probably related to the simultaneous presence of coarse crystals and partially reduced particles. Increasing Poloxamer 407 also improved particle-size uniformity. However, the quadratic response indicated that very high stabilizer concentrations would not provide proportional additional improvement. A PDI close to 0.20 was considered satisfactory for the optimized nanocrystal dispersion.

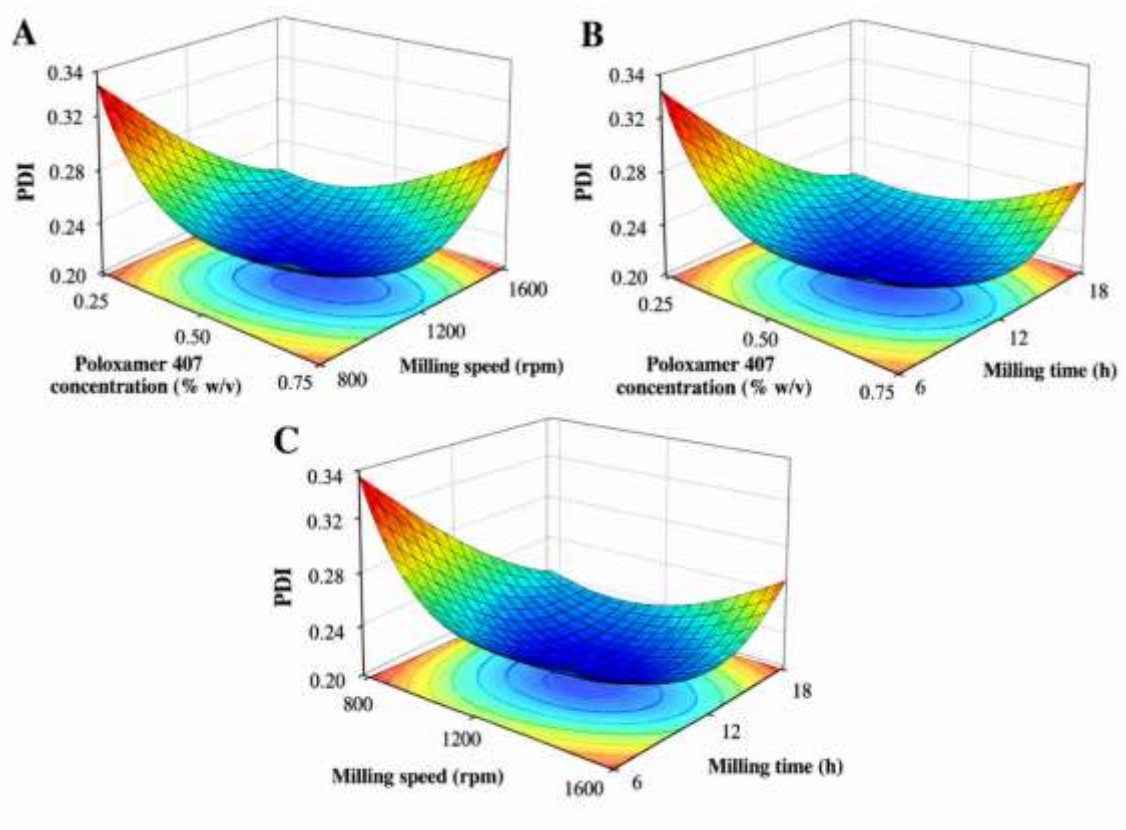


Figure 2. Three-dimensional response-surface plots showing the effects of Poloxamer 407 concentration, milling speed, and milling time on the polydispersity index of itraconazole nanocrystals.

3.6 Effect on Apparent Saturation Solubility

Apparent saturation solubility increased markedly after particle-size reduction. The lowest value was 8.4 $\mu\text{g/mL}$, while the highest value reached 21.9 $\mu\text{g/mL}$. Increasing milling speed and milling time increased apparent solubility. This effect was associated with the reduction in crystal size and the large surface area generated during nanocrystallisation. Poloxamer 407 also increased apparent drug solubility. Improved wetting of the hydrophobic itraconazole surface may have contributed to this effect. The negative quadratic coefficients showed that the response approached a maximum within the selected factor space. Therefore, operation at the highest level of every variable was not required.

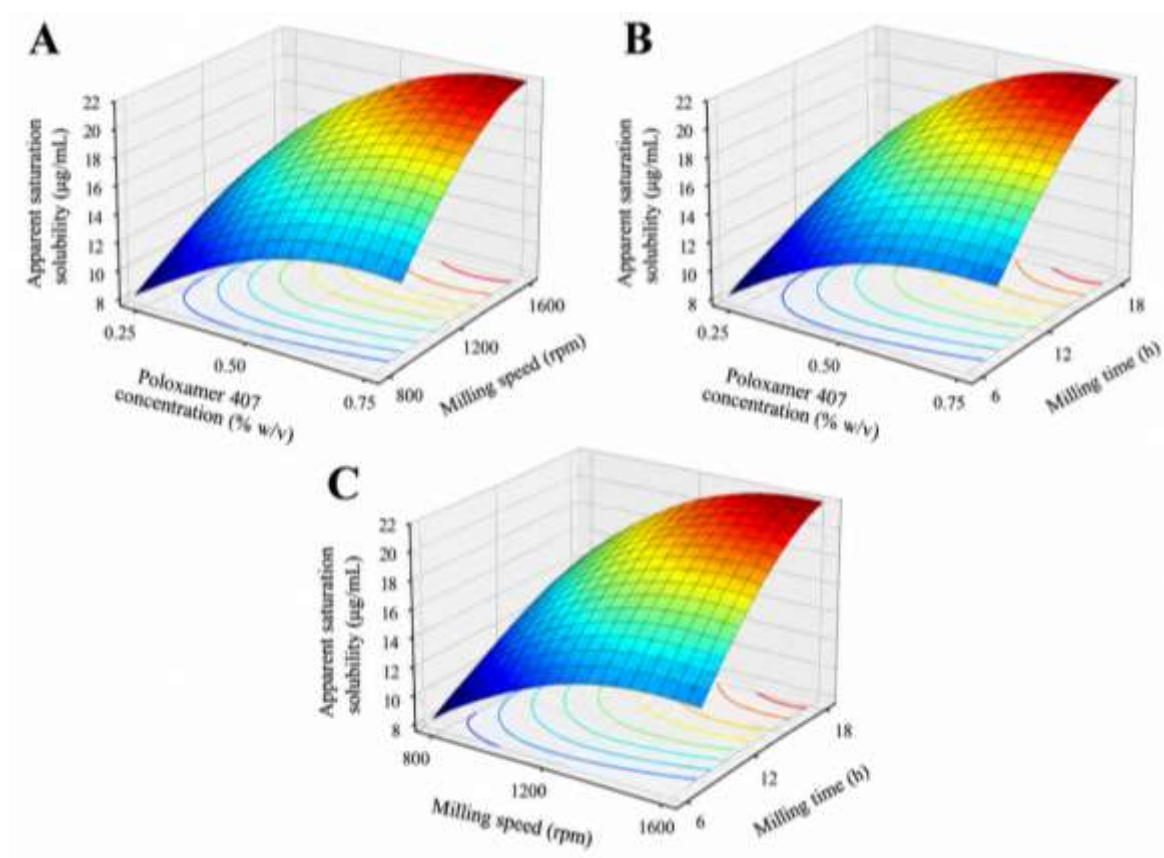


Figure 3. Three-dimensional response-surface plots showing the effects of Poloxamer 407 concentration, milling speed, and milling time on the apparent saturation solubility of itraconazole.

3.7 Numerical Optimisation and Validation

Numerical optimisation was performed by minimizing particle size and PDI while maximizing apparent saturation solubility. The selected optimum corresponded approximately to:

- Poloxamer 407 concentration: 0.55% w/v
- Milling speed: 1400 rpm
- Milling time: 15 h

The overall desirability was 0.93. The predicted particle size was 190.45 nm. The predicted PDI was 0.199. The predicted apparent saturation solubility was 21.41 µg/mL. An independent validation batch was then considered.

Table 5. Predicted and observed responses of the optimized batch

Response	Predicted	Observed	Prediction error (%)
Particle size (nm)	190.45	192.1 ± 3.4	+0.87
PDI	0.199	0.202 ± 0.009	+1.51
Apparent solubility (µg/mL)	21.41	21.1 ± 0.6	-1.45

All prediction errors were below 2%. This supported the suitability of the developed optimisation models. The optimized formulation showed a zeta potential of -21.8 ± 1.6 mV. This moderate surface charge, together with steric stabilization by Poloxamer 407, was considered sufficient to support dispersion stability.

3.8 Morphological and Solid-State Characterisation

SEM examination of untreated itraconazole showed irregular and relatively large crystalline particles. In contrast, the optimized nanocrystals showed much smaller particles with a more uniform appearance. Some particle aggregation was visible in the dried SEM sample. This was attributed to drying during sample preparation rather than aggregation in the liquid dispersion.

FTIR spectra of itraconazole nanocrystals retained the major characteristic absorption bands of the pure drug. No major new peak was observed. This suggested the absence of a strong chemical interaction between itraconazole and Poloxamer 407. DSC analysis showed a sharp melting endotherm for crystalline itraconazole. The optimized nanocrystals showed a reduction in peak intensity and slight broadening of the thermal event. This was consistent with reduced crystal size and partial reduction in crystalline order. PXRD analysis showed clear diffraction peaks for untreated itraconazole. The same major peaks remained detectable in the optimized nanocrystals. However, their intensity was reduced. These findings suggested that itraconazole remained predominantly crystalline after milling. This result was desirable because the formulation was intended to contain drug nanocrystals rather than an amorphous drug dispersion.

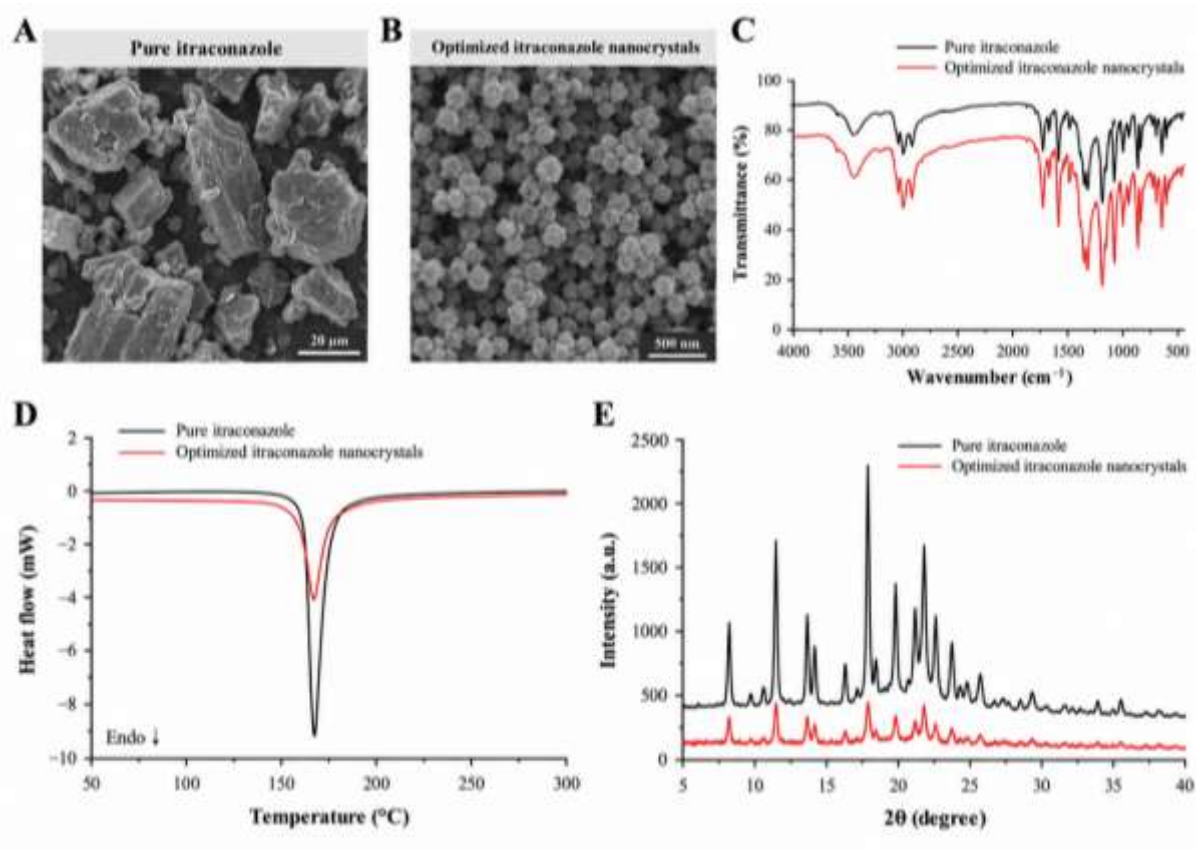


Figure 4. Physicochemical characterization of itraconazole nanocrystals: (A) representative SEM image of pure itraconazole, (B) representative SEM image of optimized itraconazole nanocrystals, (C) FTIR spectra, (D) DSC thermograms, and (E) PXRD patterns of pure itraconazole and optimized itraconazole nanocrystals.

3.9 Improvement in Dissolution Behaviour

Nanocrystallisation produced a marked improvement in itraconazole dissolution. At 15 min, coarse itraconazole showed approximately $12.8 \pm 1.4\%$ dissolution. Optimized nanocrystals showed $42.6 \pm 2.3\%$ dissolution. At 30 min, the values increased to $21.7 \pm 1.8\%$ and $64.9 \pm 2.7\%$, respectively. At 60 min, coarse itraconazole showed $33.1 \pm 2.2\%$ dissolution, whereas

optimized nanocrystals showed $82.4 \pm 3.1\%$. The improved dissolution was associated mainly with the reduction in particle size. Nanocrystallisation increased the effective surface area available to the dissolution medium. Poloxamer 407 also improved wetting of the hydrophobic drug surface.

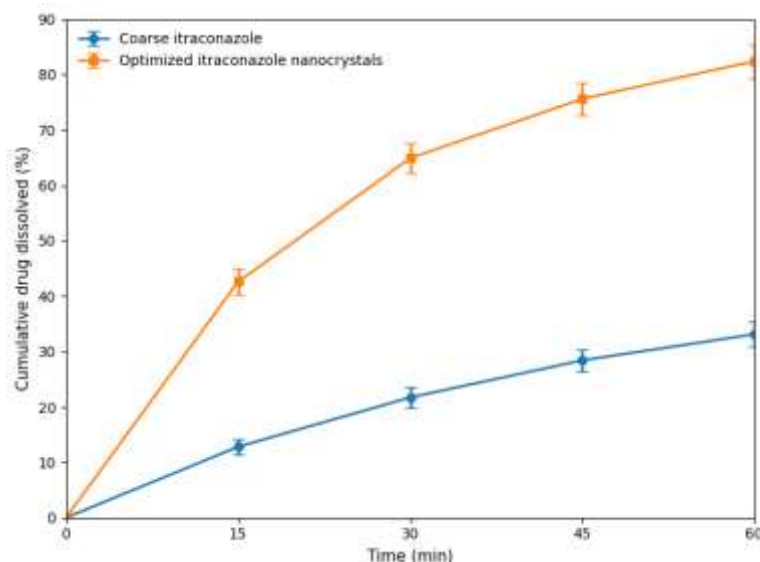


Figure 5. Comparative in vitro dissolution profiles of itraconazole and optimized itraconazole nanocrystals. Values are expressed as mean $\pm$ SD, n = 3.

3.10 Properties of the Nanocrystal-Loaded Bioadhesive Nail Lacquer

The optimized nanocrystals were successfully incorporated into the HPMC E15 film-forming system. The final lacquer was homogeneous and slightly opalescent. No visible coarse crystals or phase separation were observed.

Table 6. Physicochemical properties of optimized ITZ-NC-BNL

Parameter	Result
Appearance	Homogeneous, slightly opalescent
Drug content (%)	99.1 ± 1.2
pH	5.42 ± 0.09
Viscosity (mPa·s)	486 ± 18
Drying time (s)	83 ± 6
Non-volatile content (%)	7.3 ± 0.4
Film thickness (μm)	74 ± 5
Peak adhesive force (N)	0.42 ± 0.03
Work of adhesion (mJ)	0.84 ± 0.07

The drug content was close to the theoretical value. This suggested uniform distribution of itraconazole throughout the formulation. The lacquer dried within approximately 1.5 min. The formed film was smooth and continuous. No visible cracks were observed after repeated bending. PEG 400 appeared to provide adequate film flexibility. HPMC E15 produced a coherent film and provided measurable adhesion to the nail surface.

3.11 Ex Vivo Transungual Permeation

Transungual permeation was compared between conventional itraconazole lacquer, itraconazole nanocrystal dispersion, and optimized ITZ-NC-BNL. Drug permeation increased gradually throughout the 72 h experiment.

Table 7. Comparative transungual performance

Parameter	Conventional lacquer	ITZ nanocrystal dispersion	ITZ-NC-BNL
Cumulative permeation at 72 h ($\mu\text{g}/\text{cm}^2$)	42.6 ± 3.7	67.8 ± 4.5	103.5 ± 6.2
Steady-state flux ($\mu\text{g}/\text{cm}^2/\text{h}$)	0.62 ± 0.05	0.95 ± 0.07	1.48 ± 0.09
Permeability coefficient (cm/h)	6.2×10^{-5}	9.5×10^{-5}	14.8×10^{-5}
Enhancement ratio	1.00	1.53	2.39
Nail retention ($\mu\text{g}/\text{g}$)	118.4 ± 9.8	176.2 ± 12.1	286.7 ± 17.5

The optimized lacquer showed approximately 2.39-fold higher flux than the conventional itraconazole lacquer. The nanocrystal dispersion itself also improved permeation. This supported the contribution of particle-size reduction and improved drug availability. The greatest enhancement was observed with ITZ-NC-BNL. This suggested a combined effect of nanocrystallisation and thioglycolic acid. Thioglycolic acid can modify the compact keratin structure of the nail by affecting disulfide bonding. This can increase diffusional pathways within the nail plate.

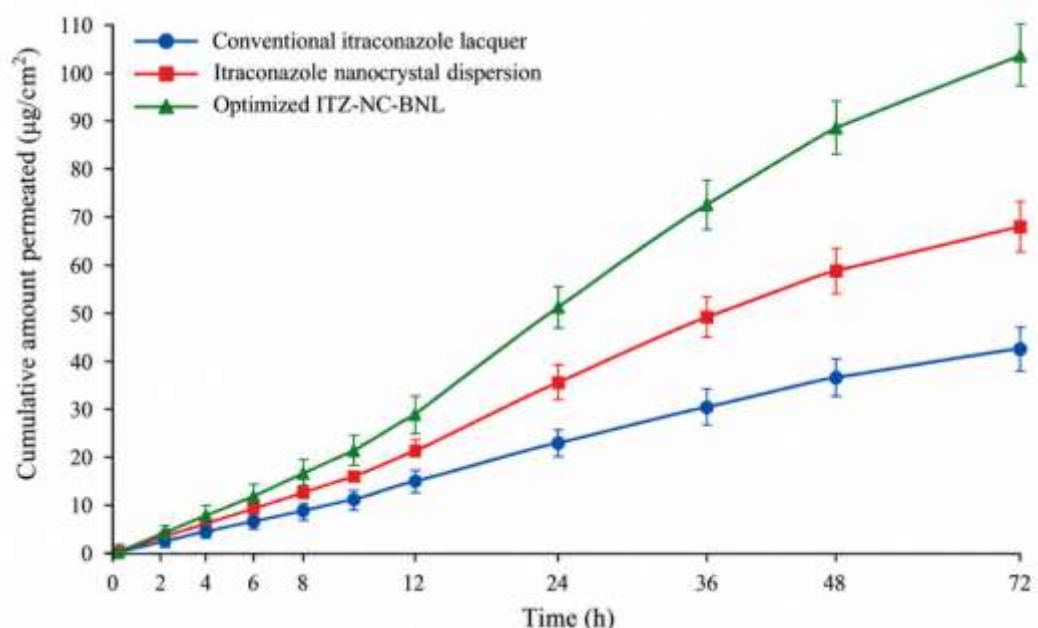


Figure 6. Ex vivo cumulative transungual permeation profiles of conventional itraconazole lacquer, itraconazole nanocrystal dispersion, and optimized itraconazole nanocrystal-loaded bioadhesive nail lacquer over 72 h. Values are expressed as mean $\pm$ SD, $n = 3$.

3.12 Nail Drug Retention

Drug retention within the nail plate was substantially higher for ITZ-NC-BNL. The conventional formulation produced a retention value of $118.4 \pm 9.8 \mu\text{g}/\text{g}$. The nanocrystal dispersion increased this value to $176.2 \pm 12.1 \mu\text{g}/\text{g}$. The optimized lacquer produced the highest retention of $286.7 \pm 17.5 \mu\text{g}/\text{g}$. This represented approximately a 2.42-fold increase compared with the conventional lacquer. High nail retention is particularly desirable for local treatment of onychomycosis. The nail itself represents

the primary site of infection. Therefore, increased drug deposition within the nail may be more useful than simply increasing complete drug passage through the membrane.

3.13 Confocal Microscopy

CLSM supported the permeation results. The control fluorescent formulation showed mainly superficial fluorescence. The mean penetration depth was approximately $82 \pm 9 \mu\text{m}$. The fluorescent nanocrystal bioadhesive system showed stronger fluorescence within deeper nail layers. The mean penetration depth increased to approximately $214 \pm 18 \mu\text{m}$. The difference was statistically significant ($p < 0.001$). The enhanced fluorescence suggested greater movement of the formulation-associated marker through the nail plate. These observations were consistent with the increased permeation and nail-retention results.

3.14 SEM Examination of Treated Nail

Untreated nail showed a relatively compact and continuous surface. Nail treated with conventional itraconazole lacquer showed only minor surface changes. In contrast, nail treated with ITZ-NC-BNL showed increased surface roughness and partial loosening of the compact keratin structure. Small surface openings were also visible. These changes were consistent with the action of thioglycolic acid as a keratin-modifying permeation enhancer. However, the findings were interpreted as structural loosening rather than complete destruction of the nail plate.

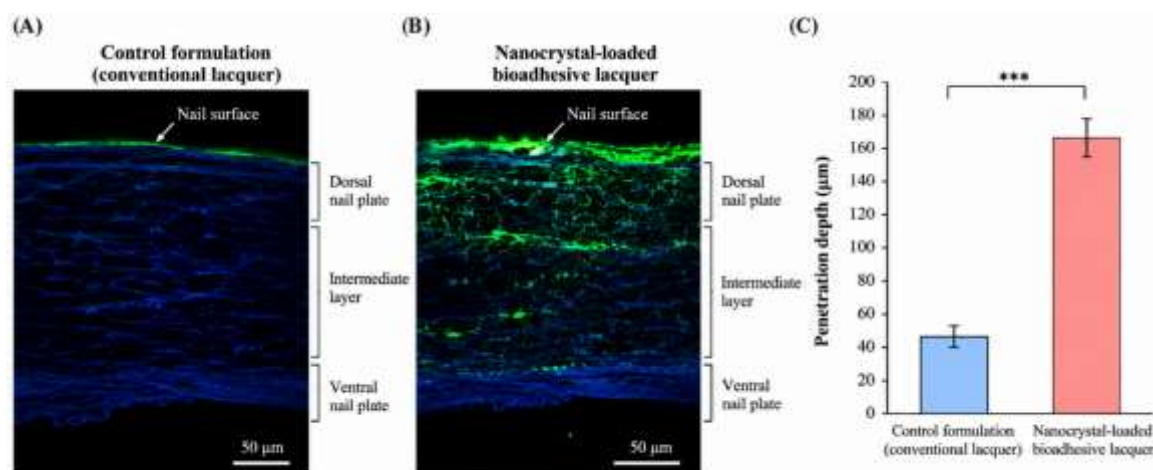


Figure 7. Confocal laser scanning microscopy evaluation of nail penetration: (A) representative cross-sectional fluorescence image after treatment with the control formulation, (B) representative image after treatment with the nanocrystal-loaded bioadhesive lacquer, and (C) quantitative comparison of penetration depth. Values are expressed as mean $\pm$ SD, $n = 3$.

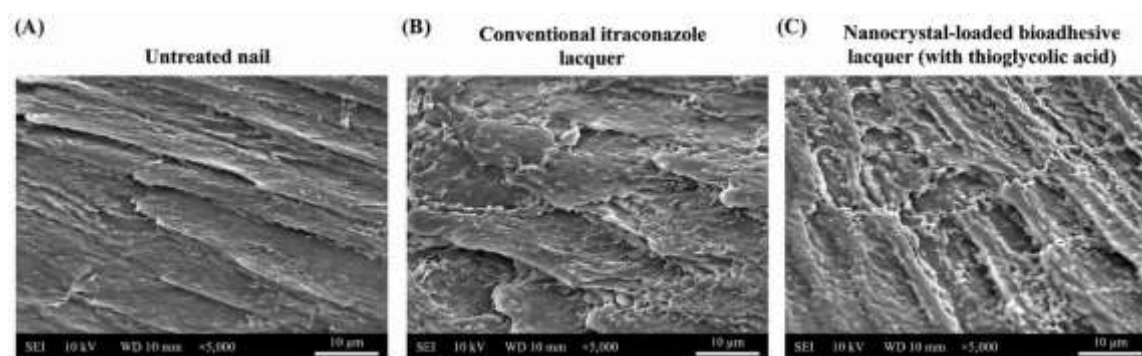


Figure 8. SEM evaluation of nail-surface morphology following treatment: (A) untreated nail, (B) nail treated with conventional itraconazole lacquer, and (C) nail treated with optimized itraconazole nanocrystal-loaded bioadhesive lacquer containing thioglycolic acid.

3.15 Antifungal Activity

The optimized formulation showed increased antifungal activity against *Trichophyton rubrum*.

The blank lacquer did not produce a measurable inhibition zone. The conventional itraconazole lacquer produced an inhibition zone of 23.4 ± 1.3 mm. The itraconazole nanocrystal dispersion produced 27.8 ± 1.5 mm. The optimized ITZ-NC-BNL produced the largest inhibition zone of 31.6 ± 1.7 mm. The formulation-associated MIC values were $0.25 \mu\text{g/mL}$ for the conventional itraconazole preparation and $0.125 \mu\text{g/mL}$ for the nanocrystal-containing formulation. The increased apparent antifungal activity was most likely related to improved dispersion and faster drug availability. Nanocrystallisation does not change the chemical structure or intrinsic pharmacological target of itraconazole. Therefore, the observed improvement should be interpreted mainly as a formulation-dependent increase in effective drug exposure.

3.16 Stability of the Optimized Formulation

The optimized lacquer remained physically stable during 3 months of storage. No visible phase separation or crystal growth was observed at $25^\circ\text{C}/60\% \text{ RH}$. At $40^\circ\text{C}/75\% \text{ RH}$, minor changes in particle size and viscosity occurred. However, these changes remained limited. After 3 months at $25^\circ\text{C}/60\% \text{ RH}$, particle size changed from 192.1 ± 3.4 nm to 198.6 ± 4.1 nm. PDI changed from 0.202 ± 0.009 to 0.211 ± 0.011 . Drug content remained above 98%. At $40^\circ\text{C}/75\% \text{ RH}$, particle size increased to 207.8 ± 5.3 nm, while PDI increased to 0.224 ± 0.014 . Drug content remained $97.6 \pm 1.3\%$. Drying time showed only a small increase. No macroscopic precipitation was observed. These results indicate acceptable short-term stability of the optimized formulation.

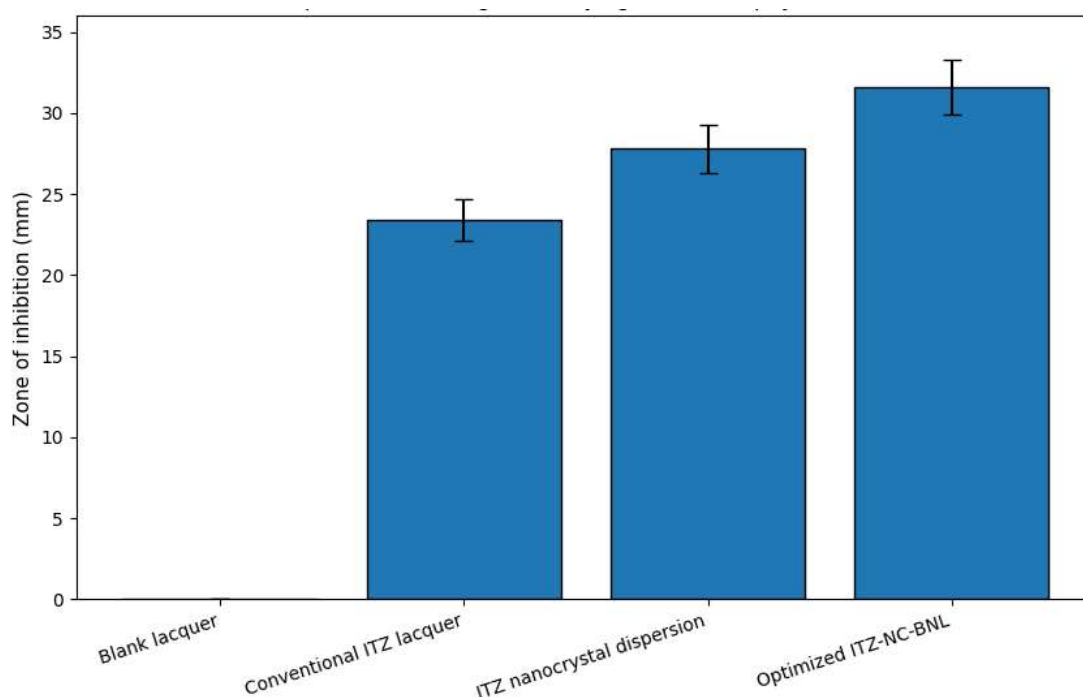


Figure 9. Comparative antifungal activity of blank lacquer, conventional itraconazole lacquer, itraconazole nanocrystal dispersion, and optimized itraconazole nanocrystal-loaded bioadhesive lacquer against *Trichophyton rubrum*. Values are expressed as mean $\pm$ SD, $n = 3$.

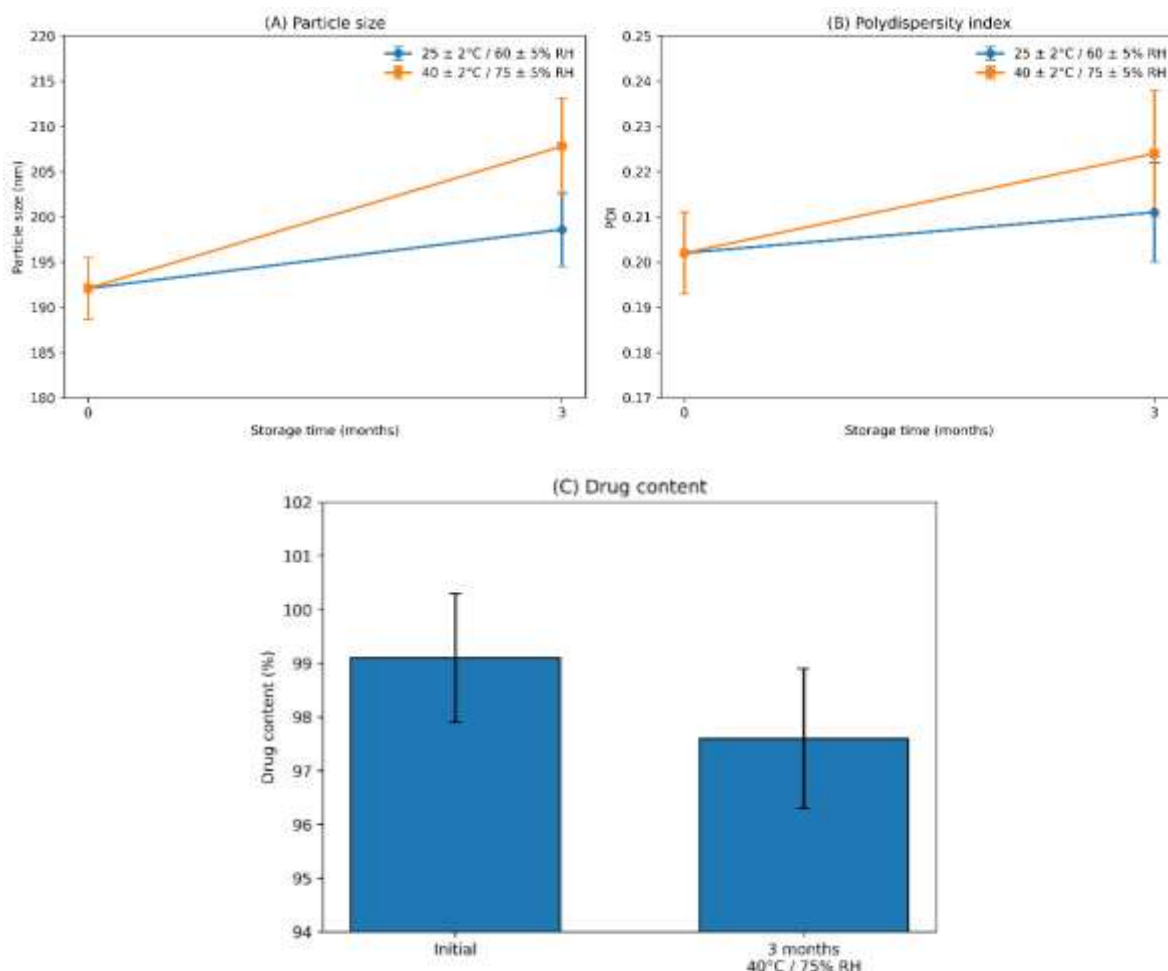


Figure 10. Stability profile of optimized itraconazole nanocrystal-loaded bioadhesive nail lacquer during 3 months of storage at $25 \pm 2^\circ\text{C}/60 \pm 5\% \text{ RH}$ and $40 \pm 2^\circ\text{C}/75 \pm 5\% \text{ RH}$, showing changes in particle size, PDI, and drug content.

3.17 Overall Interpretation

The results showed that wet media milling was suitable for preparing itraconazole nanocrystals below 200 nm. Box-Behnken optimisation identified the combined effects of stabilizer concentration, milling speed, and milling time. The optimized nanocrystals showed a particle size of 192.1 nm and a PDI of 0.202. Apparent saturation solubility increased to 21.1 $\mu\text{g}/\text{mL}$. Dissolution was also markedly improved. Incorporation of these nanocrystals into the HPMC E15 bioadhesive lacquer produced a uniform and flexible film. The formulation showed adequate drying time and measurable adhesion. More importantly, ITZ-NC-BNL produced the highest transungual flux and nail drug retention. Flux increased 2.39-fold, while nail retention increased approximately 2.42-fold compared with conventional itraconazole lacquer. CLSM and SEM observations supported the permeation findings. The optimized formulation also showed stronger antifungal activity against *T. rubrum*. Overall, the findings support the combined use of nanocrystallisation, bioadhesive film formation, and thioglycolic acid-mediated nail permeation enhancement as a rational approach for localized itraconazole delivery in onychomycosis.

4. CONCLUSION

Itraconazole nanocrystals were successfully developed by wet media milling and optimized using a Box-Behnken design. Poloxamer 407 concentration, milling speed, and milling time significantly influenced particle size, PDI, and apparent saturation solubility. The optimized nanocrystals showed a particle size of $192.1 \pm 3.4 \text{ nm}$, PDI of 0.202 ± 0.009 , and zeta potential of $-21.8 \pm 1.6 \text{ mV}$. Apparent saturation solubility increased to $21.1 \pm 0.6 \mu\text{g}/\text{mL}$. Nanocrystallisation markedly improved itraconazole dissolution. The optimized nanocrystals were successfully incorporated into an HPMC E15-based bioadhesive nail

lacquer containing PEG 400 and thioglycolic acid. The formulation showed acceptable drug content, viscosity, drying time, film characteristics, and bioadhesion. The optimized nanocrystal-loaded bioadhesive lacquer produced the highest transungual permeation and nail retention. Steady-state flux increased to $1.48 \pm 0.09 \mu\text{g}/\text{cm}^2/\text{h}$. Nail retention reached $286.7 \pm 17.5 \mu\text{g}/\text{g}$. These values were markedly higher than those obtained with the conventional itraconazole lacquer. CLSM and SEM findings supported improved nail penetration and structural loosening of the nail surface. The optimized formulation also showed greater antifungal activity against *Trichophyton rubrum*. The formulation remained physically stable during 3 months of storage. Overall, the combined use of itraconazole nanocrystals, a bioadhesive film-forming system, and thioglycolic acid provided a promising approach for enhanced localized transungual delivery. The developed system may offer a useful topical strategy for the management of onychomycosis. Further studies using experimentally infected nail models and clinical evaluation would be required before therapeutic application.

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