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Hansen Solubility Parameters and QbD-Oriented HPLC Method Development and Validation for Dermatokinetic Study of a Miconazole-Loaded Cationic Nanoemulsion in Rat Model

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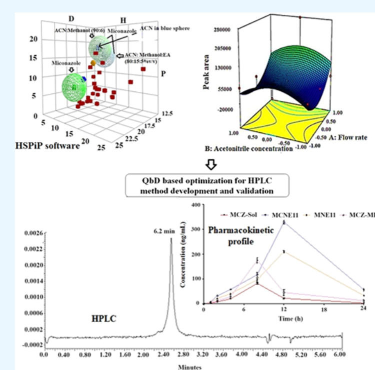
ABSTRACT: Miconazole (MCZ) is a potential antifungal drug to treat skin infections caused by *Candida*, *Tinea pedis* (athlete's foot fungal infection), *Tinea cruris* (jock itching in the groin and buttocks), and *Tinea corporis* (red scaly rash on the skin). The current study focused on Hansen parameter-based solvent selection (HSPiP software) and method development optimization using an experimental design tool for sensitive, accurate, reproducible, economic, rapid, robust, and precise methodology to quantify MCZ in rat plasma. Moreover, a Taguchi design was used for screening two independent factors (flow rate and ACN content). Quality by design (QbD) was employed to optimize and identify the right ratio of mobile phase composition and its impact on the peak and retention time. The elution of MCZ was achieved using methanol and acetonitrile (15:85 v/v ratio) at a retention time of 6 min and optimal flow rate (1 mL/min). Finally, the method was validated based on accuracy, precision, linearity, selectiveness, and high recovery at varied concentrations as per the International Council for Harmonization (ICH) guidelines. The method was linear (r^2 = 0.999) over the explored concentration range (250−2000 ng/mL) at 270 nm detection wavelength. The optimized method was used to quantify in vivo pharmacokinetic (PK) study after transdermal application of MCZ-loaded formulations (MCNE11, MNE11, MCZ-Sol, and MCZ-MKT). HSP-oriented solvent selection and quality by design-based optimized process variables and composition in the optimized analytical methodology were quite convincing and have been a cutting-edge MCZ analysis method so far. The validated method was robust, economic, and rapid with high specificity and selectivity.



INTRODUCTION

Miconazole nitrate (MCZ) is a clinically available imidazole compound introduced to the market more than 40 years ago. The drug can be used for parenteral delivery (0.2−1.2 g thrice a day), topical as cream, lotion, spray liquid, and spray powder (2%), and vaginal as pessaries (2% once a day for 7−14 days) in the treatment of systemic (candidal) and local (*Tinea pedis*, *Tinea cruris*, and *Tinea corporis*) fungal infections.[1,2] However, intravenous use has been limited due to toxicity associated with the product.[1] The drug is primarily used to treat cutaneous mycoses and related fungal infections as describe before (*Tinea cruris*, *Tinea pedis*, and vulvovaginitis).[2] MCZ represents milestones in the management of dermatomycoses and related conditions.[3] Considering ongoing findings in the mechanisms of MCZ action, the drug plays a pivotal role in topical treatments of superficial skin fungal infections. Adverse reactions consist of burning, itching, stinging, and erythema on the treated site.[4] In our previous published report, we had developed cationic nanoemulsion for topical delivery of miconazole to treat topical and dermal fungal infection with augmented permeation and improved efficacy by establishing synergism with the excipients possessing innate antifungal potential.[5] An attempt has been made to develop and validate high-performance liquid chromatography (HPLC) methodology for simplicity, high sensitivity, and accuracy for drug quantification from rat skin tissue samples.

The bioanalytical method is well known to quantify the drug from biological samples such as urine, serum, plasma, and tissue homogenates using HPLC due to high sensitivity, specificity, accuracy, and precision.[6] This technique is commonly employed to quantify MCZ from tissue/biological samples for various purposes such as pharmacokinetic assessment, biological metabolites, and in vivo toxicity in explored animal models or clinical study. Furthermore, HPLC enables the drug to be quantified rapidly from the desired biological samples without sample degradation. Implementing new programs for selection of the right solvents and their combination is now gaining attention for its robustness, reliability, reproducibility, and simplicity with high sensitivity.

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For this, Hansen solubility parameter (HSP)-based selection of the mobile phase can be a promising approach for the method development and validation of HPLC for quantification of MCZ from tissue samples. Therefore, we applied HSPiP software to estimate the HSP of the drug and solvents. The program provides a series of solvents and their combination ratios for maximum drug elution depending upon the physicochemical properties and cohesive energies of substances. Furthermore, Design Expert (also called as QbD) is a well-known tool for its robust and reliable methodology by identifying critical factors to have significant impact on the desired analysis outcomes such as peak area and peak retention time.[7] Thus, the combined effort of HSPiP and QbD was the new state of the art for the strategic method development with high robustness and reproducibility for the product scale up from the laboratory scale to the industrial outcome. The method may be economic, simple, and low energy-based to revalidate and reproduce for the bioanalytical method.[8]

Various analytical methodologies have been implemented to quantify the drug from biological samples such as gas chromatography (GC), HPLC, high-performance thin-layer chromatography (HPTLC), and spectrophotometry using organic and inorganic solvents.[9−12] No report has been published for the quantification of MCZ in plasma/tissue samples using the combined strategic approach (HSPiP and QbD programs) for the mobile phase selection and optimization so far. We addressed the application of the HSPiP program for the selection of the suitable solvent for the mobile phase preparation followed by QbD-based optimization. The developed method was suitable, simple, and rapid for the estimation of pharmacokinetic parameters such as the maximum plasma concentration (C_{max}), the time required to reach C_{max} (T_{max}), and the area under the curve (AUC) for the explored drug after topical application. This enables us to understand the relative drug access in the skin and plasma after topical application for tangible MCZ monitoring.

■ MATERIALS

Miconazole nitrate (as MCZ, 98.0% pure) was gifted from "Velite Pharmaceuticals, Ludhiana, Punjab, India". The reagents (disodium hydrogen phosphate dehydrate, $Na_2HPO_4 \cdot H_2O$, and potassium hydrogen phosphate, $KHPO_4$) for the preparation of phosphate buffer (pH 6.8) were purchased from Merck, Mumbai, India. Various HPLC-grade organic solvents (methanol, ethyl acetate, acetonitrile, and ethanol) from purchased from SD Fine, Mumbai, India. Milli-Q water was used an aqueous solvent for the method development (Millipore, Molsheim, France).

■ METHODS

Estimation of Hansen Solubility Parameters (HSP) for Mobile Phase Selection. Miconazole (MCZ) is chemically a potential azole (imidazole) to treat fungal infection (*mucocutaneous candidiasis*, *oropharyngeal candidiasis*, and *vaginal candidiasis*) with few reported activities against Gram-positive bacteria. The drug is associated with low molecular weight (416 g/mol), high lipophilicity ($\log P = 6.1$), and poor aqueous solubility (0.0007 mg/mL). Pharmacologically, MCZ inhibits ergosterol synthesis of the fungal cell wall. The drug has 0 and 2 hydrogen bond donor count and hydrogen bond acceptor count, respectively.[13] Several topical creams and related products are available in the market with limited therapeutic efficacy due to poor drug solubility and permeation across the skin barrier. Thus, selection of a suitable solvent, surfactant, lipid, and cosurfactant could be a promising approach to formulate an alternative transdermal product with anticipated permeation and therapeutic benefits after topical application. In the preliminary study, selection of excipients and the subsequent optimization process led to several benefits such as reduced time of product development, reduced product cost, high patient compliance, and high regulatory acceptance. For this, the predictive program (Hansen solubility parameter, HSP) and Design Expert (experimental design tool) played a vital role during product development. HSPiP software is conceptually based on the total energies of the drug and solvent. Compounds possessed total cohesive energy distributed as polarity energy, dispersion energy, and hydrogen bond formation ability energy.[14,15] Mathematically, this was expressed below as eq 1

$$\delta^2 = [\delta_d]^2 + [\delta_p]^2 + [\delta_h]^2 \quad (1)$$

where δ_d, δ_p, and δ_h are the solvent dispersion power, the solvent polarity (due to the dielectric nature), and the solvent hydrogen bond formation capability, respectively. Hansen estimated δ_p and δ_h values of a polymer in a targeted solvent by an empirical method (experimentally) to get the best "volume". They were adopted as three coordinates of axes for developing a three-dimensional solubility sphere with radius "Ro". The program classified "good" or "bad" solvent depending upon the position of the solvent in a three-dimensional solubility sphere. A solvent falling within the sphere or on the surface is considered "good" and vice versa.[14] Various input parameters were used to run the program obtained from the literature, by default, and the experiment. Then, targeted HSP values of the combined ratio of two solvents were estimated at a given radius and 100% check value (composition not exceeding 100%). Theoretically, the difference of any HSP between the drug and solvent should be close to zero or zero for maximum solubilization in the studied solvent or binary mixture at fixed temperature (δ_d of solute − δ_d of solvent ∼ zero, δ_p of solute − δ_p of solvent ∼ zero, and δ_h of solute − δ_h of solvent ∼ zero).[14,15] We explored various transdermal and nontopical products with improved drug solubility and high permeability by implementing HSP (estimated from the program). In the program, various trials were run to select a suitable composition of solvents and their ratios for maximum drug solubility. During screening, HSP values of the drug and HSP values of human skin were taken into consideration for developing the mobile phase and the drug extraction from skin, respectively. The HSP of human skin was obtained from the literature as presented in the Results and Discussion section.[14,15]

Experimental Solubility of Miconazole. The drug was solubilized in various organic solvents and a combination of solvents. The HSPiP program was used to predict various organic solvents based on HSP parameters. The program suggested various combinations. Therefore, these were taken into account for the experimental solubility at 40 °C (at column temperature). Briefly, a precise amount of MCZ was transferred to a glass vial containing the solvent. The mixture was shaken for 72 h to establish an equilibrium. The mixture was removed and filtered to get a filtrate. The drug was estimated using a double-beam spectrophotometer (UV-1601, Shimadzu, Japan) at 270 nm. The study was replicated.

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Selection of the Mobile Phase for HPLC. Based on HSP values and experimental drug solubility, it was required to screen a right solvent, a combination of solvents, and their ratios. The drug is highly lipophilic and insoluble in water. Therefore, the program predicted no impact of water on the overall relative energy difference (RED) value. The RED value is an indicative parameter to classify a solvent as "good" or "bad". RED value < 1.0 was considered "good" solvent and vice versa. It is obvious that a solvent or combination of solvents must be suitable for the drug solubility and elution in HPLC with a RED value less than 1. Second, experimental solubility data would confirm real quantitative solubility of the drug. Thus, the predicted HSP, RED, and experimental solubility data would be considered deciding factors for the solvent selection of the HPLC mobile phase.

Chromatographic Conditions Based on HSP Values and Experimental Solubility. Based on the HSP and experimental values of the drug in the targeted solvent combination, ACN and methanol were selected as the final mobile phases for HPLC analysis. Acetonitrile and methanol were the two prime components in the mobile phase. A relatively high content of acetonitrile in the binary composition was selected based on the RED value. RED was found to decrease with an increase in the ACN content. Several trial runs were carried out using acetonitrile, methanol, and water. Water had no negative impact on the retention time and peak height. Therefore, the predicted combination of ACN and methanol (85:15) was used for the method development. The HPLC system consisted of a separation module, an autosampler, a thermostat, and a 2998 photodiode array (PDA) detector.[16] The HPLC method development and validation were carried out using a C_{18} column (250 mm × 4.6 mm, 5 μm; Waters, India).[17] The composition of the mobile phase was acetonitrile (ACN): methanol in 85:15 ratio operating in isocratic mode based on the predicted HSP. The MCZ was quantified at 270 nm.[18] Furthermore, the Empower-2 program (Waters Corporation, Milford, Massachusetts) was used to process the analytical data.

Sample Preparation (Extraction Method). Four transdermal products (already reported in our previous publication) were used to investigate the dermatokinetic study in Wistar rats.[5] We aimed to develop an HPLC methodology for efficient quantification of MCZ from the treated rat tissue with high sensitivity, reproducibility, and robustness. Therefore, we made an attempt to explore new ratios and components of the mobile phase using the Taguchi model (screening of variables), HSPiP software (screening of solvents), and Design Expert (optimization). All rats were (250−300 g) acclimatized with proper food and water for the dermatokinetic study following the approved protocol (KSU-SE-20-65) by King Saud University, Riyadh, Saudi Arabia. The rats were kept under fasting conditions for 12 h before the commencement of the experiment and dosing. They were grouped and labeled accordingly. The blood sample (2 mL) was withdrawn from the retro-orbital area, and the collected sample was centrifuged at 5000 rpm for 10 min to get the supernatant. A freshly prepared working standard solution of the drug was used (in acetonitrile). Moreover, a content of MCZ was spiked in the plasma for a working calibration curve by proper dilution (200 ng−2000 ng/mL).[17] Analysis was carried out with an injection volume of 10.0 μL for each sample. Furthermore, quality control (QC) samples were prepared by dilution with the optimized mobile phase.[18]

Preoptimization Study Using the Taguchi Model. The Taguchi model scrutinized a set of variables to minimize the variability during the analysis process. Various factors (7) and levels (2) of the Taguchi model (as shown in Table 1) were applied to identify prime factors affecting MCZ analysis in HPLC. These are flow rate, injection volume, column temperature, ACN concentration, autosampler temperature, pH of the mobile phase, and concentration of methanol at different levels such as the lowest level (−1) and the highest level (+1). These critical factors affect peak resolution in the biological tissue sample. Half normal and Pareto charts identified the most significant factor among them for validation.[19,20] Chemically, MCZ is an imidazole derivative with high lipophilicity (log $P \sim 6.25$) and solubility in alcohol (methanol) and ACN.[13] Therefore, we considered aforementioned deciding factors in the preliminary screening executing remarkable impact on the sample process development and validation for high accuracy, sensitivity, and reproducibility. In initial screening, the ACN concentration and flow rate were the prime factors affecting peak resolution and retention time. Other chromatographic attributes (injection volume, pH, methanol concentration, and injection volume) had the least or no influence on the characteristic peak. Table 1 summarizes various studied factors and responses as input parameters in the Taguchi model.

Table 1. Several Critical Parameters (Preoptimization) in Taguchi Model[a]

factors	responses	
	Y_1 (peak retention time, min)	Y_2 (peak area, mAU)
A (volume of injection, μL)	−1	−1
B (mobile phase flow rate, μL/min)	−1	+1
C (working temperature of HPLC column, °C)	+1	−1
D (acetonitrile content, %)	+1	+1
E (autosampler temperature, °C)	−1	−1
F (pH)	−1	+1
G (methanol content, %).	−1	−1

[a]Note: (A) injection volume (μL), (B) flow rate (μL/min), (C) HPLC column temperature (°C), (D) ACN concentration (%), (E) autosampler temperature (°C), (F) pH, and (G) methanol content (%). −1: low level, +1: high level.

Optimization Study. Screening study revealed that the two critical factors (flow rate as A or X_1 and ACN concentration as B or X_2) were selected for further optimization at three levels such as low (−), medium (0), and high (+) in the central composite design (CCD). The two factors at three levels expressed as Y (response) = $(3)^2$ against two responses were employed in Design Expert (Stat-Ease Inc., 1300 Godward St NE #6400, Minneapolis, Minneapolis 55413) to investigate the impact of the selected factors (from HSPiP and Taguchi model) on the targeted responses (retention time as Y_1 and peak area as Y_2). These two factors significantly influence responses/attributes for the development of robust bioanalytical method for quantification of MCZ in plasma by optimizing ACN concentration at the explored working conditions.[21−25] The target goals were minimum and maximum for Y_1 and Y_2, respectively. Eq 2 includes the studied factors (X_1 and X_2) and coefficients (β_1, β_2, β_0, β_3, β_4, and β_5) to understand the impact of the factors on the respective

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response, wherein negative and positive terms indicate the antagonistic and synergistic effect on the response, respectively.

$$Y(\text{independent variable}) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 X_1^2 + \beta_5 X_2^2 \quad (2)$$

where β_0, β_1, and β_2 are the intercept, the coefficient of X_1, and the coefficient of X_2, respectively. β_3 is the coefficient of interaction between X_1 (A) and X_2 (B), and β_4 and β_5 are the coefficients of quadratic terms for X_1 and X_2, respectively.

HPLC Method Validation. It was required to develop and validate the optimized process variables following the US FDA guidelines that complements the International conference on Harmonization (ICH) guideline Q2 (R1) for the product and the process development using HPLC.[26] The recommended parameters to be evaluated are linearity, specificity, limits of detection, and quantification such as the lower limit of detection (LLOD) and the lower limit of quantification (LLOQ). To fully understand the impact of prime factors in the method on the analysis procedure, a systemic approach using QbD (an experimental design) was required to get a robust analytical method with high sensitivity and system suitability with the explored detection and quantification limit.[27]

Linearity and Range. It is noteworthy that the statistical procedure and parameters are used to evaluate the validation characteristics based on sound principles such as analysis of variance (ANOVA) and regression analysis (correlation coefficient) for linearity. These statistical methods rely on the population normality. The linearity of the proposed method is its ability (within the provided range) to test the result, which is considered proportional to the concentration of the drug in the sample. For this, a known amount of the drug was spiked into the plasma followed by serial dilution to get a range of concentrations (250−2500 ng/mL). Each concentration was passed through a membrane filter (0.2 μm) prior to injection. A working standard curve was established by plotting the peak area (area under curve: AUC) versus the respective concentration. Under the optimized operating chromatographic conditions, the peak area was achieved in proportion to the known concentration of each sample. Regression analysis established good linearity as evidenced with the measured regression coefficient value > 0.99. The range is the concentration between the lowest and highest concentrations of the investigated drug in the tested plasma to ensure the suitability level of accuracy, precision, and linearity. Data were processed using Origin software (trial version) for statistical analysis.

System Suitability Studies. Once the procedure is successfully validated, it should remain fit and reproducible during the life cycle of the product development and the analytical process. Therefore, it is important to perform at regular intervals to evaluate the need for optimization as part of or the whole analytical procedure. The adopted optimized method should be re-evaluated and revalidated after the repeated adjustment to the operating conditions to meet the system suitability requirements. This was conducted by assessing the peak area for the MQC sample (1000 ng/mL) without plasma. The AUC value was compared with the MQC injection obtained after spiking in the plasma (n = 3).[9]

Method Specificity. It is important to revalidate that the adopted analytical method maintained its critical characteristics for performance throughout the life cycle of the product development and analysis in terms of specificity, accuracy, and precision. Therefore, the drug-free plasma samples were prepared by dilution with the mobile phase (ACN/methanol, 85:15 ratio), followed by direct filtering the samples into HPLC vials. Each sample was properly injected (10.0 μL) and monitored for any possible interference in peaks demonstrated by MCZ.[21]

Plasma Recovery. For the recovery estimation, % drug recovery was determined at LQC (500 ng/mL), MQC (1000 ng/mL), and HQC (1500 ng/mL) by comparing the peak area ratios of the extracted MCZ with that of the unextracted samples (blank).[28] Each sample was replicated for the mean and SD (standard deviation) values (n = 3).

Method Sensitivity. The values of the LLOQ and LLOD were estimated using various concentrations of MCZ in the plasma in order to inspect the signal-to-noise (S/N) ratio. The following (eqs 3 and 4) were used to estimate these parameters following the ICH guidelines.

$$\text{LOD} = 3.3 \times \sigma/\text{SD} \quad (3)$$

$$\text{LOQ} = 10 \times \sigma/\text{SD} \quad (4)$$

wherein "σ" is the SD of the Y-intercept of the regression line and SD represents the slope of the calibration curve.

Accuracy and Precision. The accuracy of the bioanalytical method indicates the closeness of agreement between the estimated values for acceptance or rejection of the validated method. Precision indicates the closeness of agreement between series of estimations through multiple sampling from the same biological sample homogenate at the same operating conditions. It was investigated using homogeneous plasma samples at three levels (repeatability, intermediate precision, and reproducibility). Repeatability is an intra-assay precision estimated for 3 days (interday) and the same day (intraday) by spiking MCZ in the plasma. %RSD of the estimated known concentration (LQC, MQC, and HQC) was used as a parameter of precision. Moreover, both parameters were estimated at intervals of 4 h (intraday) on the same day (n = 3) for QC, MQC, and HQC. %RSD of QC, MQC, and HQC values was used as an index of precision with the acceptance limit of less than 2.[11]

Robustness. The robustness is considered as a validation parameter to measure the capacity to remain unchanged by minute changes in the method parameters of the bioanalytical procedure evaluated by varied chromatographic conditions to provide its reliability. These variations were the mobile-phase composition and flow rate, and their effects were inspected for each response (Y_1 and Y_2) at three varied QC samples (500, 1000, and 1500 ng/mL).

Stability in the Plasma. It is mandatory to understand the stability of the drug in the plasma. Therefore, the drug concentration (400, 800, and 1600 ng/mL) spiked in the plasma was assayed to validate the proposed method by characterized short-term stability at 28.0 ± 2 °C for 2 h, freeze−thaw cycles (5 °C) for 24 h, and long-term stability (30 days) at −20 °C. The stability was also examined for the three freeze−thaw cycles. Y_1 and Y_2 were remained unchanged with no observed degradation during the study period.

Nanoemulsion Formulations (MCZ Cationic Nanoemulsion and MCZ Nanoemulsion) for Transdermal Delivery. As mentioned before, we reported in vitro and in vivo behaviors of the MCZ-loaded nanoemulsion and cationic

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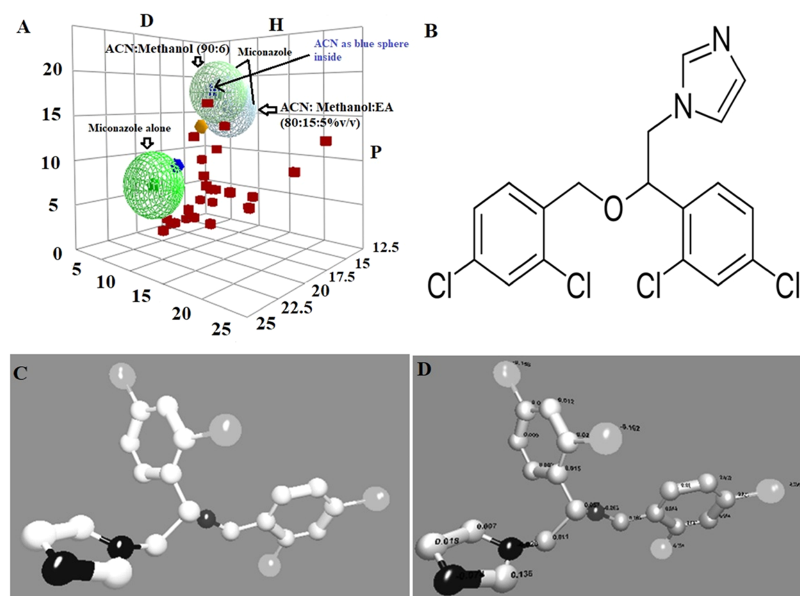

Figure 1. (A) Three-dimensional HSP of miconazole, space parameter, and combination of binary or tertiary solvent for the drug solubility, (B) structure of MCZ, (C) three-dimensional ball−stick structure of MCZ, and (D) three-dimensional ball−stick structure of MCZ with charge obtained from HSPiP software.

Table 2. Hansen Solubility Parameters of MCZ and Predicted Mobile Phase Composition (Hansen Solubility Parameters) (RED = R_s/R_o)[c]

drug and solvents	HSP values estimated							
	δ_d	δ_p	δ_h	δ_t	Rs	MVol	RED	δHD/A
miconazole[a]	20.7	6.5	3.9	22.1	18.1 (Ro)	300.7		1.4/3.6
human skin[d]	17.6	12.5	11.0					
human skin[e]	17.5	8	8					
methanol	14.4	12.3	21.3	29.9		41		14.4/14.5
ethanol[a]	15.6	9.3	17.2	25		58.2		12.5/11.3
ethyl acetate (EA)[a]	18.2	5.1	7.1	18.5			0.58	
acetonitrile (ACN)[b]	15.3	16.6	8.3	24.3		53.4	0.67	4.2/11.3
water[a]	15.5	16.0	42.0	47.6		18	2.83	
	calculated HSP in various systems using the HSPiP program							
predicted compositions	δ_d	δ_p	δ_h	HSP distance			RED	
ACN (94%) + methanol (6%)	15.3	17.7	7.1	15.9			0.88	
ACN (60%) + methanol (40%)	15.1	15.7	12.6	17			0.94	
ACN (80%) + methanol (20%)	15.2	16.9	9.3	16.1			0.89	
ACN (50%) + methanol (50%)	15.0	15.2	14.2	17.6			0.97	
methanol (60%) + ACN (40%)	14.9	14.6	15.8	18.4			1.02	
methanol (15%) + EA (5%) + ACN (80%)	14.5	15.9	7.5	16			0.88	
chemical	SMILES							
miconazole	c1 cm^3(c(cc1Cl)Cl)COC(Cn2ccnc2)c3ccc(cc3Cl)Cl							
acetonitrile	CC#N							
methanol	CO							
ethyl acetate	CCOC(=O)C							
water	HO							
ethanol	CCO							

[a]Literature values. [b]https://www.hansen-solubility.com/HSP-science/sphereVR.php. [c]Calculated value. R = The distance between the three-dimensional coordinates of the HSP for the investigated solvent and the solute (MCZ). [d][14]. [e][15]. MVol = molecular volume, RED = relative energy difference.

nanoemulsion after topical application onto rat skin and their permeation profile. It was required to develop and validate a HPLC method for the rat skin tissue to quantify the drug content and permeated drug concentration for the same formulations at high sensitivity, simplicity, and reproducibility.[5,29] These reported formulations were designated as MCNE11 (miconazole cationic nanoemulsion), MNE11 (miconazole nanoemulsion), MCZ-MKT (marketed gel), and MCZ-Sol (miconazole drug suspension). Nanoemulsions were composed of biocompatible lipid (peceol) and labrasol as a hydrophilic surfactant (hydrophilic lipophilic balance, HLB = 13.2). Propylene glycol served as a cosurfactant of both

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nanoemulsions (MCNE11 and MNE11). MCZ was solubilized in a 5% Tween 80 suspension to use as a control for comparative assessment.

Quantification of MCZ in Rat Plasma (An In Vivo Study). The present study was purposefully designed to develop and validate a robust bioanalytical method for the determination of in vivo pharmacokinetic parameters. For this, approved rats were randomly grouped for MCZCNE11, MCZNE11, MCZ-MKT, and MCZ-DS gel. Each group contained three rats. A dorsal area (3 cm^2) on each rat was properly shaved to apply (1 mg/0.5 mL) the formulations (MCZCNE11, MCZNE11, MCZ-MKT, and MCZ-DS gel).[5,29] The blood (0.5 mL) sample was withdrawn from the retro-orbital plexus at different time points (0.0, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, and 24.0 h). Each sample was ultracentrifuged to a clear supernatant. Then, the drug was quantified using the validated HPLC method. Various PK parameters were estimated.

Statistical Analysis. Various statistical parameters were calculated using software and Microsoft Excel. The study was repeated for the mean and standard deviation at $n = 3$. A value was considered significance at $p < 0.05$. The experimental design program (version 6.0.0, Sate-Ease Inc.) and GraphPad Prism (a trial version) provided various statistical parameters such as ANOVA (analysis of variance), regression coefficients, adjusted, and actual correlation coefficients.

■ RESULTS AND DISCUSSION

Estimation of the HSP for Mobile Phase Selection. The program was used to estimate various HSP values of the drug and solvents, as shown in Figure 1A and Table 2. The drug is highly lipophilic in nature with only two hydrogen bond acceptor counts (Figure 1B,1C). The HSPiP program predicted the charged location on each atom of the drug for ionic interaction with the solvent (Figure 1D). The estimated values of δ_d, δ_p, δ_h, and δ_t are 20.7, 6.5, 3.9, and 22.1 $MP^{1/2}$, respectively, as shown in Table 2. These values for solvents are presented in Table 2, wherein ethyl acetate (EA) and acetonitrile were expected to be suitable for drug solubility/miscibility. This prediction was rationalized based on the HSP of MCZ and ACN/EA. The estimated values of δ_d, δ_p, and δ_h were 18.2, 5.1, and 7.1 $MP^{1/2}$, respectively, for EA, whereas these values for ACN were found to be 15.3, 16.6, and 8.3 $MP^{1/2}$, respectively. The difference of these HSP values between MCZ and EA/ACN is minimum as compared to other solvents. However, the HSPiP program predicted various solvent combinations and their ratios for maximum MCZ solubility, as shown in Table 2. The HSP values and RED were considered to select the best combination. It is obvious from Table 2 that two combinations were found to be suitable based on minimum RED values (0.88), such as "ACN + EA + water" and "ACN + methanol". Moreover, the predicted RED for various combinations of "ACN + methanol" showed that the increased relative content of acetonitrile in the binary mixture resulted in decreased RED values. This suggested that the drug was solubilized preferably by acetonitrile compared to methanol in the binary mixture, which may be due to hydrogen bond acceptor counts in the drug interacting (δ_h, H-interaction) more with acetonitrile than with methanol.[14,15] Moreover, this impact may interfere with analytical HPLC peak resolution, peak area, and retention time. The prediction can be correlated to the reported finding, wherein authors reported that acetonitrile improved the peak and retention time when the acetonitrile concentration was relatively increased in the combination with methanol.[21] Increasing the relative content of methanol caused peak broadening and low peak intensity for MCZ estimation using HPLC from the biological sample. The EA-containing combination was dropped from selection due to some drawbacks associated with the solvent such as high UV cutoff of wavelength (256 nm) and instability with basic/acidic drug.[19,20] Thus, the predictive model is quite simulative to the previous report. Therefore, the selected solvents were acetonitrile and methanol in 85:15 ratios for further studies.

Considering other aspects such as the drug extraction from rat tissue using methanol and acetonitrile, it is prudent to correlate the estimated HSP value of the binary combination "ACN + methanol in 94:06 ratio", as shown in Table 2. The combination HSP values are quite close to the human skin (normal and abnormal). Thus, the mobile phase containing these combinations could be substantial for the drug extraction and subsequently estimation using the HPLC method. Thus, the program is quite interesting to use in the preliminary stage of the analysis method development from biological samples due to the economic process, rapid screening, and reproducible method.[14,15] The targeted HSP values of the combination were estimated at a given radius (R_O = 18.1) and 100% check value (composition not exceeding 100%).

Experimental Solubility of Miconazole. The experimental solubility of MCZ was obtained in various solvents as predicted in HSPiP. The result is exhibited in Figure 2. The

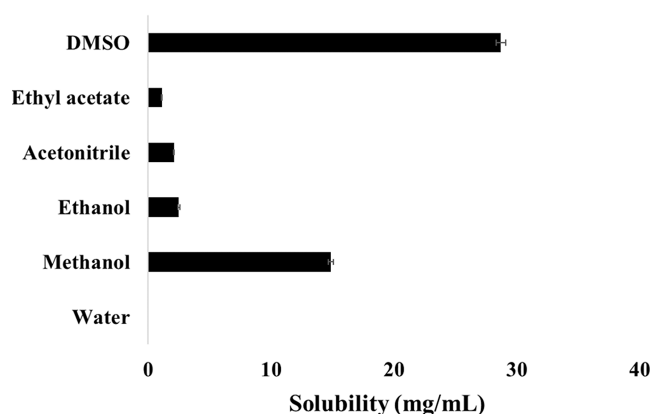

Figure 2. Experimental solubility of MCZ in various organic solvents.

solubility values of MCZ were found to be 0.00083 ± 0.00001, 14.9 ± 0.21, 2.5 ± 0.08, 2.1 ± 0.04, 1.1 ± 0.031, and 28.0 ± 0.39 mg/mL in water, methanol, ethanol, acetonitrile, ethyl acetate, and dimethyl sulfoxide (DMSO), respectively (Figure 2). Methanol and acetonitrile are classified as polar and apolar protic organic solvents, respectively. The relative polarity values of methanol and acetonitrile are 0.76 and 0.46, respectively. This may be a probable reason for the higher solubility of MCZ in methanol than in ACN. The drug was maximally solubilized in DMSO, whereas water exhibited the least solubility at the explored temperature.

Selection of the Mobile Phase for HPLC. As discussed before, the present study focused on implementing HSPiP and experimental solubility data to screen suitable solvents and the combination. The conceptual background of HSP parameters is based on the physicochemical properties of the solute and solvent. Therefore, HSP values predicted the probable

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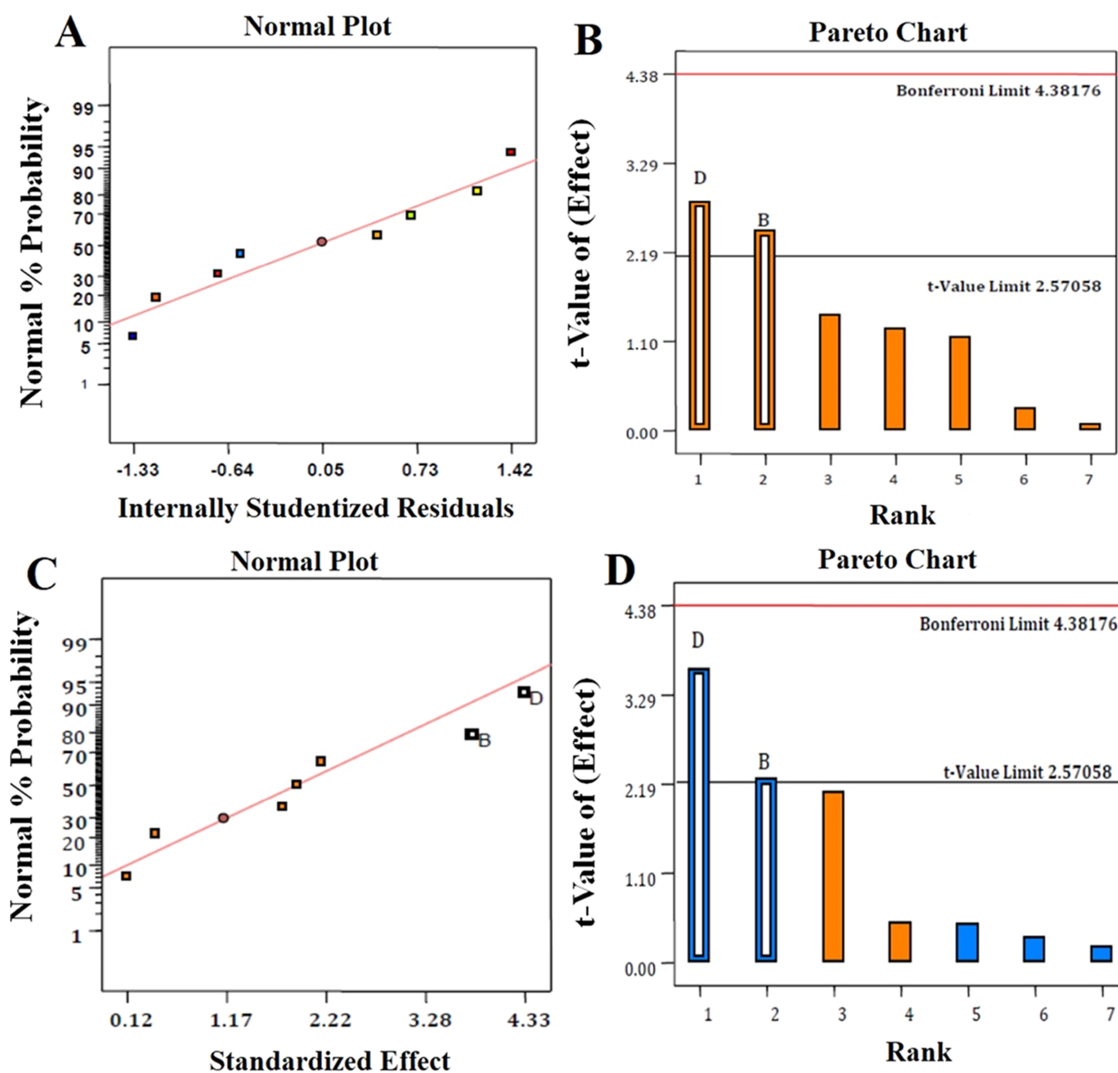

Figure 3. Half-normal and Pareto charts depicting the significant effect on bioanalytical attributes: (A) normal plot of retentional time; (B) Pareto chart of retentional time; (C) normal plot of peak area; and (D) Pareto chart of peak area in the chromatogram.

Table 3. Summary Statistical Parameters Obtained after Optimization Using CCD, Their Factor Responses, and Levels with the Provided Constraints (Goal)

dependent variables (factors) and levels			independent variables (responses, Y_1 and Y_2)	goal
design run	A (X_1) flow rate	B (X_2) CAN concentration		
1	1	−1	peak retention time (min) (Y_1)	minimum
2	−1	1		
3	0	1	peak area (mAU) (Y_2)	maximum
4	1	0		
5	0	0		
6	−1	−1		
7	1	−1		
8	1	0		
9	0	1		
10	−1	0		
11	0	0		
12	0	−1		
13	1	1		

	levels and statistical values					
responses (parameters)	low (−1)	middle (0)	high (+1)	model	p value	R^2
A (X_1): flow rate (mL/min)	1.0	1.5	2.0	quadratic	0.0017	0.9616
B (X_2): ACN concentration (% v/v)	80	85	90	quadratic	0.0013	0.9499

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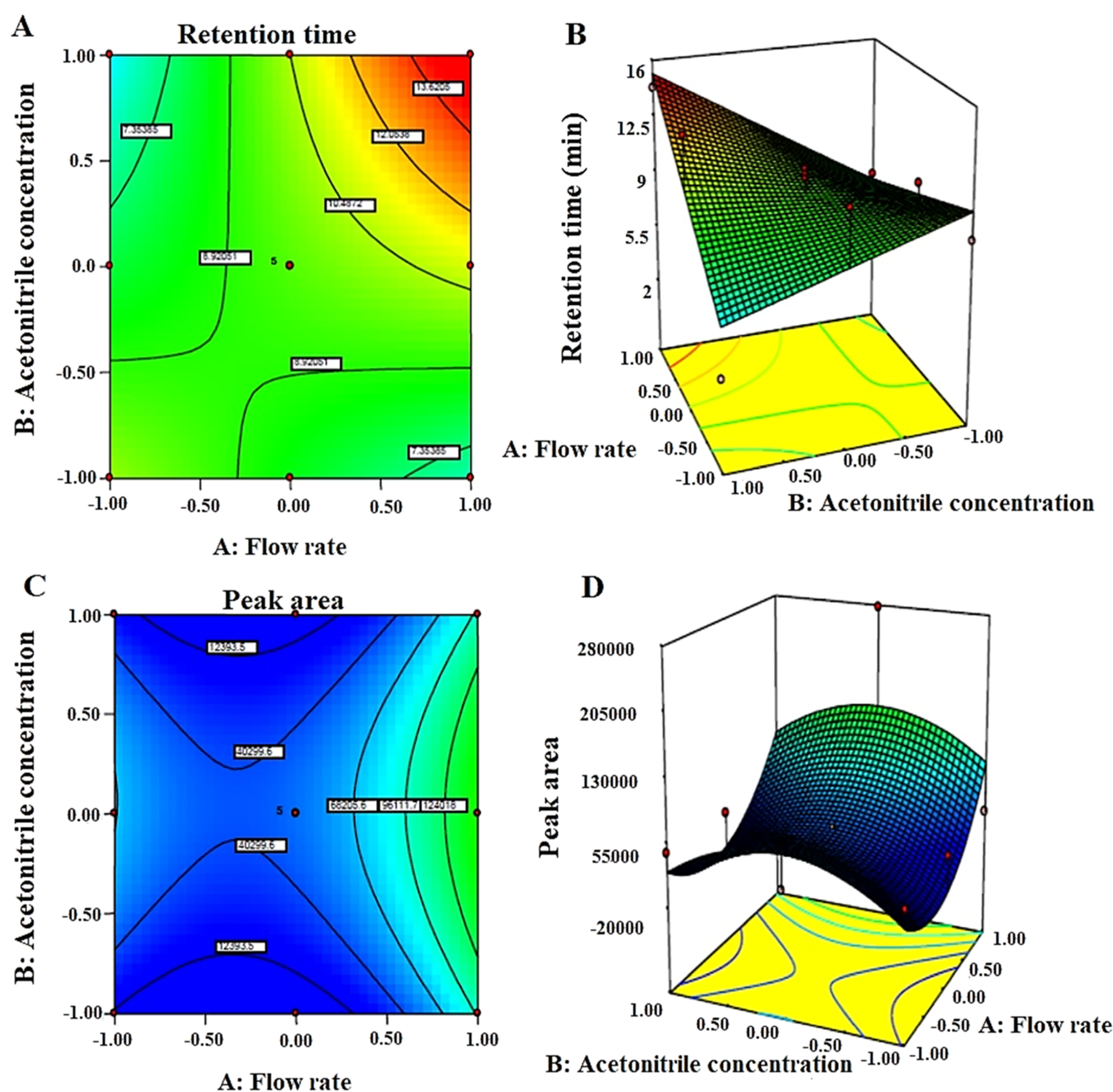

Figure 4. 2D and 3D response surface plots portraying the effect of critical process attributes (CPA) on various responses. (A) 2D plot of retention time versus factors, (B) 3D response plot of retention time versus factors, (C) 2D plot of peak area versus factors, and (D) 3D response plot of peak versus the studied factors (flow rate and acetonitrile concentration).

interaction of MCZ with the solvent based on cohesive energies. The individual HSP values of acetonitrile and methanol indicated their closeness to the drug. The experimental solubility of the drug in methanol and acetonitrile was significantly higher than in water and ethyl acetate. Ethyl acetate is generally insoluble in water and an unstable solvent compared to acetonitrile and methanol. Therefore, the ternary combination was omitted from the study. The HSP values of "δ_p" for the combined binary system "ACN + methanol" were found to be increased from 15.2 to 17.7 with decreasing relative content of methanol in the binary mixture as shown in Table 2. This might be attributed to the protic nature of both solvents in the combination system. This polarity difference had a great impact on the peak retention time and peak intensity of MCZ analysis using HPLC as reported in the literature.[21] The authors observed that retention time was reduced (10.2 min) in methanol as a mobile phase (containing 0.1% diethylamine) than acetonitrile (14.7 min) in the chromatogram.[21] The increased content of acetonitrile was reported to be beneficial in the combination due to an improved chromatogram and analysis. Methanol is a polar organic solvent responsible to interact with the stationary phase of the column through hydrogen bonding and thereby starts to compete with MCZ. This causes reduced retention time as compared to acetonitrile. However, there is a probable chance of peak broadening on increasing the relative concentration of methanol in the combination.[21] Thus, methanol and acetonitrile were selected for further optimization using least methanol in the combination.

Screening Studies (Preoptimization). Taguchi design was employed for factor screening in the development of the bioanalytical method. Results revealed from half-normal and Pareto charts indicated the influence of primarily two factors such as X_1 (flow rate) and X_2 (ACN concentration) on the studied responses (peak area and retention time). Figure 3A–D illustrates the statistically significant values of critical attributes on each of the studied responses after analysis (Table 3).

Optimization of Chromatographic Conditions. The results obtained from HSPiP and experimental solubility data were the basis for the selection of mobile phase components and their expected ratio. However, to make the method robust

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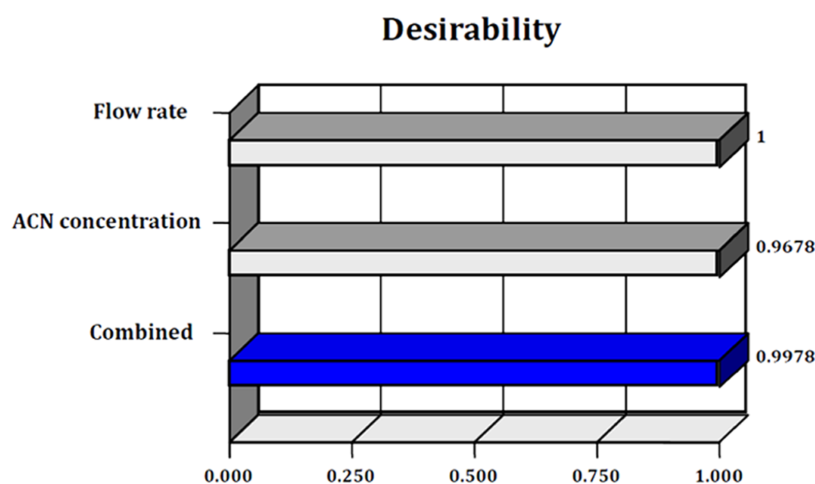

Figure 5. Desirability bar graph of the selected critical attributes (flow rate and ACN concentration).

with high reproducibility, it was required to obtain the right ratio of their components for the method development and validation for efficient quantification of the drug from the tissue sample. Therefore, HSP values and the solubility suggested that methanol and acetonitrile should be selected as major mobile phase components. From the literature and experimental HPLC runs, it was clear that acetonitrile had a major impact on the peak area and retention time. Therefore, the concentration of ACN was kept high relative to methanol. Moreover, experimental design is required to optimize a suitable ratio so that the developed method can be an optimized, reliable, sensitive, bioanalytical method for quantification of MCZ in rat plasma. The developed method can be revalidated, retransferred, and reproduced easily with high robustness. The X_1 was set between 1 and 2, whereas X_2 was selected between 80 and 90%, as presented in Tables 2 and 3 (screening). The studied responses Y_1 and Y_2 were targeted for minimum and maximum as the goal, respectively. Optimization showed 13 runs with a maximum overall desirability of 0.99. To estimate the drug content, spectral analysis of MCZ in rat plasma ranging from 200 to 400 nm showed λ_{max} at 270 nm, and the drug content was assayed at the same set absorption using a PDA detector. Considering instrumental run conditions of HPLC, the column type, column temperature, and isocratic mode of operation were kept constant for injection to avoid any interference in the studied variables.

The analysis of the interaction between factors and responses is depicted in Figure 4, wherein the 3D and 2D plots were constructed for the respective attributes. The two-dimensional (2D) surface plot and three-dimensional (3D) response surface plot for Y_1 are elicited in Figure 4A,4B, respectively. It is clear that the flow rate of the mobile phase was found to be important to have an impact on the retention time. In order to achieve minimum retention time, there must be a selection of flow rates with an optimal value. The result showed a progressive increase in retention time (RT) with an increase in the flow rate as shown in Figure 4C,4D. A robust method can be developed by selecting the flow rate with a significant ($p < 0.05$) effect on the RT. Therefore, to establish a robust method of quantification, this flow rate was optimized at a limit of 1 mL/min for the plasma samples. On the other hand, the ACN concentration also has a positive effect on the peak symmetry and peak intensity. Similarly, 2D and 3D plots for Y_1 exhibited that retention time decreased with the increase in concentration. Figure 4C,4D indicates that the peak area first decreased with an increase in the flow rate followed by an increase in peak area. Therefore, the selection of the factor must be optimal (neither high nor low). In the case of factor X_2, the peak area was first increased with the increase in acetonitrile concentration followed by reduction. Therefore, the recommended acetonitrile concentration was set at 85% and the optimized flow rate was chosen as 1.0 mL/min. The increased concentration (85% v/v) of ACN and optimal level of flow rate sharpened the peak symmetry. This predictive pattern is quite convincing with the result from the experiment and the reported literature, which may be correlated to the protic (proton donor) nature of ACN. The authors reported that the higher content of acetonitrile causes reduction in the retention time of MCZ in HPLC methodology.[21] Furthermore, one-way ANOVA of the statistical analysis was employed to analyze the best fit of the model for its suitability as summarized in Table 3. The values of p are 0.0012 and 0.00016 for X_1 and X_2, respectively, to confirm the best fit of the model adopted for analysis of Y_1 and Y_2 (Table 3). The correlation regression coefficients for Y_1 and Y_2 were obtained as 0.96 and 0.96, respectively. The purpose of the proposed bioanalytical method was to obtain an optimal and robust method, which was established by minimizing the flow rate and optimizing ACN concentration. These criteria resulted in the low value of Y_1 and the maximum value of Y_2.

The desirability numerical parameter was estimated for each factor and response between zero and unity, as shown in Figure 5. The optimization process was suitable, evidenced with the obtained desirability value between 0 and 1. The maximum overall numerical desirability (0.9978) value indicated the best fit of the model (Figure 5). Moreover, this was further supported with the estimated adjusted correlation coefficient (r^2) and the observed value (falling under close values). Moreover, the optimized chromatographic conditions for the estimation of MCZ from the plasma sample are listed in Table 4.

BIONALYTICAL METHOD VALIDATION

Linearity and Standard Working Calibration Curve. The standard calibration curve of MCZ in the rat plasma was prepared in the range of 250−2000 ng/mL with a correlation coefficient value of r^2 = 0.999. Figure 6A,B illustrates the

Table 4. Summary of the Analytical HPLC Method to Estimate MCZ in the Plasma Sample

analytical HPLC conditions	
buffer pH	4.5
mobile phase composition	ACN/phosphate buffer (85:15)
column temperature (°C)	40
column dimension	250 mm × 4.6 mm, 5 μ particle size
injection volume (μL)	10
flow rate (mL/min)	1.0
wavelength (nm)	270
run time (min)	6.0

representative chromatograms of MCZ in the optimized mobile phase and rat plasma, respectively. The regression equation for the calibration curve was $Y = 131.6x$ for MCZ. Both peaks (for both samples) were obtained at 2.6 min as shown in Figure 6A,B. The obtained peak was well resolved without significant trailing and peak splitting at the explored operating conditions.

LLOD and LLOQ. These values were estimated as 100 and 330 ng/mL for LLOD and LLOQ, respectively, suggesting the suitability and sensitivity of the proposed bioanalytical method to quantify MCZ in the rat plasma for PK study.[30]

Plasma Recovery. Table 5 summarizes the percent recovery data obtained from the plasma samples. The observed % recovery was achieved in the range of 99.1−100.7% for MCZ. The high content recovery reveals that the developed method was appropriate as per the ICH Q2 (R1) guidelines.[31]

Precision and Accuracy. The within-day (intraday) and three different day (interday) accuracy and precision of the proposed method were investigated at three different concentration levels of MCZ in the plasma measured and the result is presented in Table 6. The values of RSD% did not exceed 2.0% at all QC concentrations corroborating the high repeatability and reproducibility of the optimized bioanalytical HPLC method to estimate MCZ in the biological sample.[7] Moreover, the observed values of % accuracy were found in a range between 90.8 and 98.2% (Table 6).

Stability Study in Plasma. The results of a stability study conducted at varied temperatures and time points are presented in Table 7. Table 7 reveals a certain change in the concentration from the initial concentration (800 and 1600 ng/mL), which may be correlated to the loss of the drug during the sample process and estimation. The loss of MCZ at different circumstances was anticipated to be encountered during the sample storage, handling, and processing. The variation is within the acceptable range as evidenced with the %CV values falling within the limit (<2.0%). Comparing the result with the lowest storage temperature (−20 °C, frozen state), the loss of MCZ in the rat plasma was remarkably low (2.7%) as shown in Table 7.[32] A similar pattern of the drug loss was observed at higher concentration (1600 ng/mL), and the observed percent loss was 2.9% in the frozen state. Thus, the recommended temperature for the storage of plasma

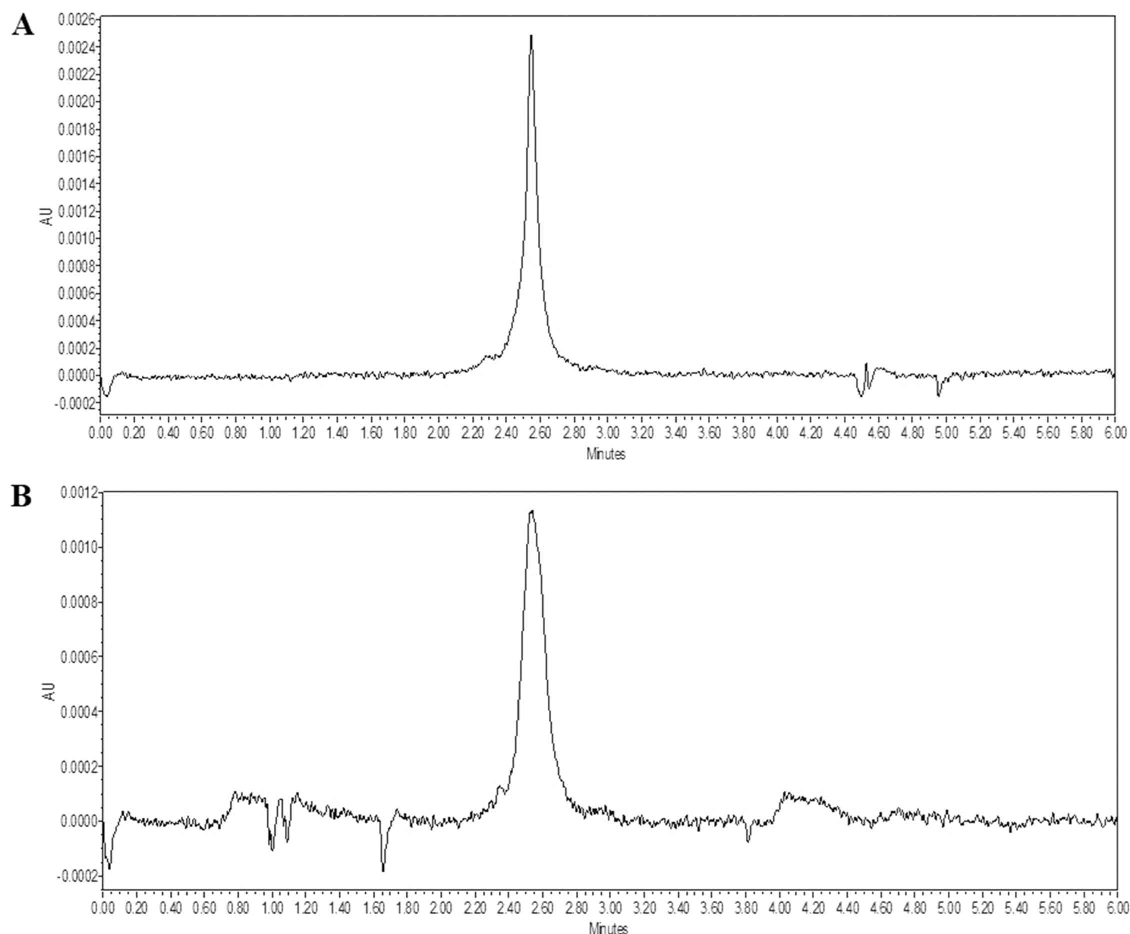

Figure 6. Chromatogram of MCZ in (A) mobile phase and (B) rat plasma of MCZ in rat plasma.

Table 5. Various Analytical Validation Parameters and Their Levels at Three QC Concentrations

nominal concentration (ng/mL)	levels (%)	theoretical concentration (ng/mL)	recovery concentration (ng/mL) (mean ± SD)	recovery	CV (%)
500	90	450.0	445.43 ± 2.64	98.8%	1.9
	95	475.0	472.21 ± 7.12	99.5%	1.5
	100	500.0	506.74 ± 10.11	101.4%	1.8
1000	90	900.0	893.72 ± 6.33	99.3%	1.3
	95	950.0	956.65 ± 7.87	100.7%	1.8
	100	1000.0	999.67 ± 5.42	99.7%	0.9
1500	90	1350.0	1368.92 ± 11.71	101.5%	1.5.
	95	1425.0	1405.04 ± 8.43	98.6%	1.7
	100	1500.0	1494.00 ± 5.54	99.58%	0.7

Table 6. Within-Day (Intraday) Accuracy and Precision Values for MCZ Estimated in the Rat Plasma[a]

concentration (ng/mL)	observed concentration (ng/mL)	% accuracy	precision (% RSD)
	intraday		
500 (LQC)	481.5 ± 4.51	96.3	1.3
1000 (MQC)	978.0 ± 7.24	97.8	1.7
1500 (HQC)	1473.0 ± 6.43	98.2	1.9
	interday		
500 (LQC)	454.1 ± 5.71	90.8	0.9
1000 (MQC)	943.2 ± 9.31	94.3	1.8
1500 (HQC)	1462.5 ± 7.27	97.5	1.2

[a]Footnote: LQC = low quantification concentration, MQC = medium quantification concentration, HQC = high quantification concentration, and RSD = relative standard deviation.

Table 7. Summary of the Stability Study Conducted at Varied Temperatures and Times

	mean	CV (%)	mean	CV (%)
	initial concentration (ng/mL)			
varied circumstances	800		1600	
3 freeze−thaw (5 °C)	712.1 ± 8.99	1.66	1441.6 ± 11.21	1.71
Short-term stability for 24 h at 30 °C	736.2 ± 7.85	1.36	1476.1 ± 14.54	1.05
Long-term stability for 30 days at frozen temperature (−20 °C)	776.3 ± 9.21	1.49	1552.1 ± 12.65	1.59

containing MCZ must be frozen temperature (low −20 °C) for maximum precision and accuracy in the validation parameters.

Formulation Developed for In Vivo Assessment. As discussed before, we reformulated cationic and anionic nanoemulsions loaded with MCZ. A summary of the composition and their characterized parameters are tabulated in Table 8. Oleylamine served as a positive charge inducer and was expected to internalize with the negatively charged cellular surface of skin for maximized adhesion and subsequent drug permeation and drug penetration via electrostatic interactions. Other variables were kept constant to investigate the impact of positively charged nanoscale globular mediated enhanced permeation as discussed in the previous report.[29] In the present study, it was required to develop an efficient, simple, and reproducible economic bioanalytical method for the quantitative estimation of MCZ from rat skin samples treated with MCNE11 and MNE11. HSPiP and QbD-oriented solvent selection for the HPLC mobile phase and identification of the prime factors affecting analytical retention time and peak were first explored for simplified bioanalysis at low cost within a

Table 8. Composition and Evaluations of the MCZ Cationic Nanoemulsion (2% w/v)

parameters	MCNE11	MNE11
peceol (lipid) (%)	18.4	18.4
Lab/PG (S_{mix})	25.0	25.0
oleylamine (0.05%)	0.05	
S_{mix} (ratio)	2:1	2:1
water (%)	56.55	56.6
globular size (nm)	145.0	137.0
PDI	0.291	0.24
ζ potential (mV)	+28.1	−30.2
% EE	89.8	85.9

short period. The prediction program assisted to opt for the desired and targeted organic solvents in proper proportion.

In Vivo Pharmacokinetic Study. The developed and validated method was successfully implemented to study PK parameters (in vivo dermatokinetics) of MCZ in rat plasma after the dermal application of the aforementioned formulations. Previously, we explored product development, product optimization, and in vitro−ex vivo studies using suitable models.[29] Furthermore, a validated method with high sensitivity and reproducibility was established to confirm localized and systemic (in vivo) performance of the developed formulations (MCNE11, MNE11, MCZ-MKT, and MCZ-Sol) for their therapeutic efficacy. We conducted PK study after dermal application, and the results are portrayed in Figure 7 and Table 9.

The results of the concentration−time profile revealed that the pharmacokinetic parameters such as C_{max} (363%), $AUC_{0\text{-}t}$ (457%), and $AUMC_{0\text{-}t}$ (867%) for MCNE11 (cationic nanoemulsion) were significantly ($p < 0.01$) high as compared to MCZ-Sol. Similarly, these parameters for MCNE11 were remarkably increased many times, C_{max} (170%), $AUC_{0\text{-}t}$ (216%), and $AUMC_{0\text{-}t}$ (342%), as compared to MCZ-MKT (C_{max}: 175.08 ± 11.7; $AUC_{0\text{-}t}$: 1244.26 ± 83.5; $AUMC_{0\text{-}t}$: 14570.76 ± 1187.4). The high values of various parameters of MCNE11 due to the positive charge on the globules increase the penetration [20]. Other pharmacokinetic parameters such as MRT, $T_{1/2}$ K_e, and T_{max} were observed to be high as compared to MCZ-Sol, MCZ-MKT, and MNE11. It is clearly evident from Figure 7 that formulations applied on the skin showed clearly varied lag time points. Maximum lag time (98 min) was observed in the case of MCZ-Sol, which may be correlated to its poor aqueous solubility at ambient and pH at the neutral side. MCNE11 and MNE11 exhibited a lag time of 25 and 36 min, respectively, for the drug permeation across rat skin due to the nanoparticle and aqueous solubility, which help

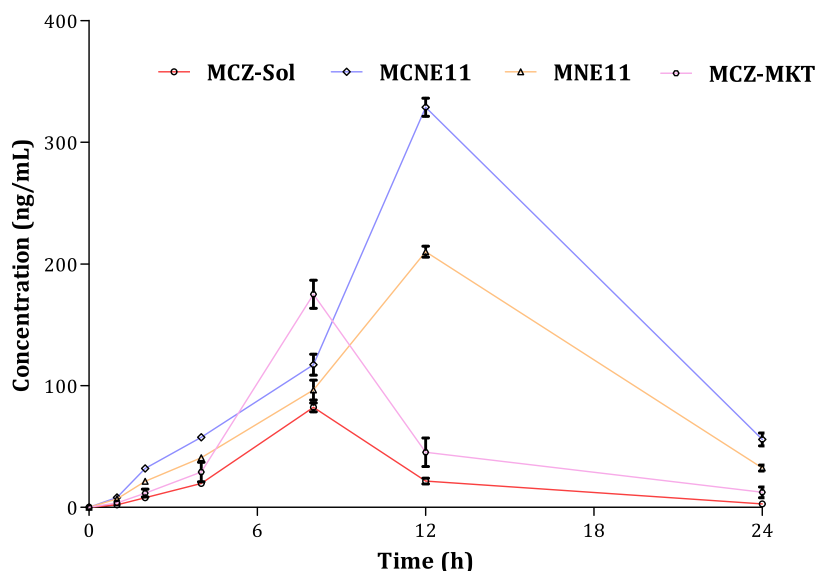

Figure 7. In vivo dermatokinetic study of various formulations (MCZ-Sol, MCNE11, MNE11, and MCZ-MKT) in the rat plasma after transdermal delivery.

Table 9. In Vivo Pharmacokinetic Parameters of MCZ-Sol, MCNE11, MNE11, and MCZ-MKT Following Application on Rat Skin (mean ± SD, *n* = 3)

pharmacokinetic parameters	MCZ-Sol	MCNE11	MNE11	MCZ-MKT
C_{max} (ng/mL)	82.15 ± 3.7	298.71 ± 3.5	210.12 ± 7.2	175.08 ± 11.7
T_{max} (h)	6.2	12.7	8.5	7.5
$T_{1/2}$ (h)	6.42 ± 0.7	15.87 ± 1.8	10.90 ± 2.6	8.09 ± 1.7
$AUC_{0-\infty}$ (ng.h/mL)	590.92 ± 29.1	2695.41 ± 45.1	1966.12 ± 90.7	1244.26 ± 83.5
AUMC (ng.h^2/mL)	5754.05 ± 327.5	49904.53 ± 1327.5	30768.76 ± 1651.2	14570.76 ± 1187.4
MRT (h)	9.51 ± 1.6	15.21 ± 2.2	13.63 ± 3.3	10.99 ± 2.8
Ke (h^{-1})	0.28 ± 0.02	0.11 ± 0.03	0.15 ± 0.02	0.21 ± 0.03

the high content of MCZ to penetrate up to the viable epidermis and dermis region.[33]

CONCLUSIONS

MCZ is a weak basic drug with variable retention time and peak pattern as reported in various studies. Therefore, it was mandatory to develop and validate a robust HPLC method to quantify the drug from the rat plasma with high accuracy, reproducibility, and simplicity. The HSPiP program has not been employed for the method development of the drug so far. Therefore, the applied HSPiP program assisted in reducing the time of solvent screening and reduced the cost burden of analysis based on the estimated RED values and HSP. The interactions among critical attributes (factors) were first determined, which affect the robustness of the HPLC method using Taguchi design. Furthermore, the QbD program predicted a right composition of the mobile phase to achieve the targeted outcome as set in the model. The study described QbD-steered method development of a sensitive, simple, accurate, and robust bioanalytical methodology for quantification of MCZ in the plasma as evidenced with high extraction accuracy (≥98% of recovery) and precision and low run time for the elution. High sensitivity of the developed HPLC method was indicated by a low LLOD value (100 ng/mL). Dermamacokinetic parameters estimated in the rat plasma indicated that the method development and validation were suitably implemented for the quantification of MCZ in plasma after transdermal application of nanoemulsions of MCZ (MCNE11 and MNE11) formulations for improved AUC and C_{max} compared to MCZ-Sol and MCZ-MKT. The validated bioanalytical method was found to be linear, accurate, specific, economic, rapid, and robust.

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Notes

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