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RESEARCH ARTICLE

Analytical Method Validation for Ketoconazole Determination in Niosomal Gel using UHPLC

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ABSTRACT:
Ketoconazole is an antifungal agent, due to its high permeability and low solubility, This drug is classed as Class II by the Biopharmaceutical Classification System (BCS). Low solubility limits penetration into the skin layers, thereby reducing its bioavailability. Niosomes are nanovesicular systems capable of delivering the ketoconazole when applied topically. Gel formulations are widely preferred for delivering active compounds topically due to their ease of application. Therapeutic effectiveness is indicated by the stability of the active compound's concentration during use. This study aims to validate the analytical method for determining ketoconazole content in Niosomal gel formulations prepared with a mixture of non-ionic surfactants. The research began with forming a ketoconazole niosome system using cholesterol and surfactants (Span 60 and sucrose palmitate ester) with varying hydrophilic-lipophilic balance (HLB) values. The optimal niosome system was achieved with a vesicle size of 353.4nm±40.5, zeta potential of -37.10mV±2.99, Polydispersity Index of 0.571, and entrapment efficiency of 99.78%±0.002. Niosomes were incorporated into Carbopol 940 gel bases at concentrations of 0.5% (F1), 0.75% (F2), and 1% (F3). The concentration of ketoconazole in the gel was then determined by ultra-high performance liquid chromatography (UHPLC). Validation conditions were optimized on a C-18 column measuring 4.6 × 150mm × 5µm at 30°C, a flow rate of 1.0 mL/min, and a mobile phase of acetonitrile-water (60:40, v/v) at pH 6.0, and UV-Vis detector at 205nm. The validation results met the criteria for selectivity, linearity, LOD, LOQ, accuracy, precision, and robustness. The ketoconazole content in the gel (F1) was 98.85% ±0.04. Study result showed that ketoconazole content in Niosomal gel can be accurately determined using a validated UHPLC method..

KEYWORDS: Ketoconazole, Niosomal gel, Validation, UHPLC.

INTRODUCTION:
Ketoconazole is an antifungal drug in the BCS Class II category, characterised by low solubility and high permeability and belonging to the imidazole derivative group with potential applications in antifungal treatments[1]. However, oral administration of ketoconazole poses a high risk of liver injury, a risk notably higher than that associated with other oral antifungals. Consequently, oral use of ketoconazole is no longer recommended, making topical administration a safer alternative. Using dermal circulation to cover the entire body and deeper tissues, topical drug administration entails moving the medication from a topical formulation to the intended local site[2].

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The low solubility of ketoconazole limits its skin penetration, thereby affecting its bioavailability. This challenge necessitates a suitable delivery system to enhance ketoconazole penetration, and thus, niosomes have been developed. Amphiphilic vesicles having a bilayer structure, niosomes, are created when cholesterol and non-ionic surfactants are hydrated together. This configuration improves drug entrapment and solubility[3]. Additionally, non-ionic surfactants used in niosome preparation have been shown to act as penetration enhancers, Damage to the stratum corneum (SC) – the skin barrier offering the greatest degree of invulnerability[4]. As a result, niosomes are a promising transdermal delivery system, facilitating the permeation and diffusion of drugs into the skin while reducing hepatic and gastrointestinal metabolism, thus avoiding common side effects of oral administration[5]

In pharmaceutical formulation development, achieving the highest-quality formulation efficiently and with minimal experimental trials is crucial[6]. To minimise the number of experiments needed and examine The link between the elements analyzed and the reactions observed, This research employed the Response Surface Method (RSM), a powerful optimization tool for drug delivery systems, as part of its methodology[7]. The RSM design includes developing polynomial mathematical equations and mapping the measured response to determine the most optimal formulation. A popular RSM design is the Central Composite Design (CCD), which has central, axial, and factorial points[5]. The effects of various formulation parameters on the properties of niosomes as an effective transdermal ketoconazole delivery system are thus examined in this work using RSM. This was accomplished by analysing factor effects and four responses using the two-factor CCD model in order to identify the best formulation: Zeta potential (ZP), particle size measurement (PS), Polydispersity Index (PDI), and entrapment efficiency percentage (EE%). After the ideal formulation was determine,ed, ketoconazole niosomes were incorporated into a gel formulation for easier application.

Gel formulations are semi-solid preparations composed of big organic moleculs or tiny inorganic particles that a liquid can penetrate[8,9]. Compared to other topical preparations, gels have advantages such as high Adhesion strength, non-clogging of pores (ensuring unimpeded pore respiration), easy washability with water, and efficient drug release[10]. The concentration of ketoconazole in Niosomal gel was established by high-performance liquid chromatography (UHPLC). The UHPLC method is a separation technique using a fixed solid phase and a mobile liquid phase. The separation process is conducted through partitioning, adsorption, or ion exchange. Common stationary phases include modified silica or polymer granules, while the mobile phase consists of solvents or solvent mixtures specified in each monograph[11,12].

The method for ketoconazole assay, including analytical method validation using UHPLC, was optimized previously.Regarding precision, the validation results comprised relative standard deviation (RSD) using a mobile phase consisting of methanol and phosphate buffer at a ratio of 30:70. testing at 0.3–0.5%, accuracy recovery at 97–100%, and a linearity correlation coefficient R^2 of 0.9996[13].

This study aims to develop an analytical method by improving the composition ratio of the mobile phase. After establishing the ideal composition of the mobile phase, the next step will be to validate this analytical method for accurately assessing the ketoconazole content in Niosomal gel formulations. Validation is a crucial step to guarantee the analytical results' dependability and accountability study validation criteria were reviewed, including linearity, selectivity, Accuracy, accuracy, limit of detection and quantification, and procedure resilience.

MATERIALS AND METHODS:

Materials:

Ketoconazole BPFI (BPOM, Indonesia), Span 60 (Sigma), sucrose ester palmitate (Compass Food), cholesterol (Nippon Fine Chemical), chloroform (Merck), methanol (Merck), phosphate buffered saline/ PBS (Solarbio), water for injection (Ikapharmindo Putramas), Nipagin M (Clariant Produkte, Switzerland), TEA (Sigma-Aldrich, USA), Carbopol 940NF (Lubrizol AM, Cleveland), propylene glycol (Clariant Produkte, Germany), Millipore membrane 0.45.

Equipment:

Ultra-high performance liquid chromatography (UHPLC) (Waters), C-18 column measuring 4.6 × 150 mm × 5µm, Particle Size Analyzer (PSA) (Delsamax Beckman Coulter), rotary evaporator (Eyela, Japan), pH meter (Hanna, USA), magnetic stirrer and glassware.

Methods:

Experimental Design:

Design Expert (Version 13) with a CCD model and two factors was used for the statistical optimisation of ketoconazole niosomes Table 4. The outcomes of this experimental design can be evaluated using this model, which also creates a mathematical relationship between independent and dependent variables. The ketoconazole niosome formulation experiments were based on the results from a previous CCD model study with two factors. using a cholesterol-to-surfactant ratio of 1: In this experimental design. the amount of cholesterol (0.1

mg – 1mg. X1) and the HLB value of the surfactant (4 – 8. X2) were used as independent variables with variations at two levels (low and high). The dependent variables included PS (Y1), ZP (Y2), PDI (Y3), and EE% (Y4).

Preparation of Ketoconazole Niosomes:
The thin film hydration approach was utilized to create ketoconazole niosomes. Surfactant and cholesterol were dissolved in 10cc of a 1:1 chloroform-methanol mixture, then combined with ketoconazole. Subsequently, the solvent was evaporated using a vacuum rotary evaporator at 150 rpm for approximately 10 minutes at a temperature of 55±2°C. A thin film of the solid mixture forms on the surface of the bottle. The dry film was hydrated with an aqueous phase (PBS 7.4). The formed multilamellar vesicles were cooled in a refrigerator. Then mixed for half an hour with a magnetic stirrer at 60°C and 50 revolutions per minute, before being subjected to 5 minutes of ultrasonic treatment at 150 Volts.

Characterization of Ketoconazole Niosomes:
To determine particle size (PS), zeta potential (ZP), and Polydispersity Index (PDI), 100 µl of ketoconazole niosomes were dispersed and diluted in 4 ml of Aqua Pro Injection. Subsequently, the sample was examined for 10 minutes using a particle analyzer.

Entrapment efficiency:
After placing 1.5 mL of the ketoconazole niosomes suspension in a microtube and centrifuging it for 60 minutes at 12.000 rpm, supernatant was gathered, The concentration of ketoconazole was measured using UHPLC at a wavelength of 205 nm.

Determination of ketoconazole concentration:
A standard solution was prepared by dissolving 10.0 milligrams of accurately weighed ketoconazole in a 10 mL volumetric flask. Add the methanol, and the solution was shaken until the substance dissolved completely, and then ultrasonicated until homogeneous. Dilution was carried out by pipetting 0.1 ml of the solution into a 10 ml volumetric flask, then adding 10.0 ml of methanol and homogenising. The solution was filtered via a 0.45 µm membrane before being injected into the UHPLC equipment.

About 50 ml of methanol P was added to the Niosomal gel containing precisely weighed ketoconazole, equivalent to 7.5 mg of ketoconazole. Stirring continued for 45 minutes, followed by ultrasonication lasting 10 minutes. Then add 2 mL of water and let the mixture cool. Then, dilute with methanol P to a total volume of 100 mL. Cool the solution for an hour at 5°C before filtering through filter paper. Pipette 1 mL of the filtrate. Dilute it with the solvent (water-methanol 2:98) and transfer it into a 10 mL volumetric flask. Before being introduced into the UHPLC apparatus, the solution was filtered using a 0.45 µm membrane.

Optimization of chromatographic conditions:
The stages of optimising chromatographic conditions were carried out on the mobile phase, solvent, and flow rate. Acetonitrile: water 0.1% pH 6 was used as the mobile phase in an experiment that involved 20 µL of a standard solution and a ketoconazole sample at a concentration of 0.01 mg/mL into the UHPLC system (Water, USA). Stationary phase: Waters C-18 column (4.6 × 150 mm, 5 µm); UV-Vis detector wavelength (λ) 205 nm; flow rate 1.0 mL/min; injection volume 20 µL; mobile phase: acetonitrile: water 0.1% pH 6.0 (60:40); solvent: methanol.

Validation of the Analytical Method of the Ketoconazole Niosomal Gel Concentration:
Validation of the method for determining ketoconazole concentration in Niosomal gel includes suitability, selectivity, linearity, LOD, LOQ, accuracy, precision, and robustness. Suitability test is determined by six injections of 20 µL of the standard solution. The reactions were noted, and each active ingredient's RSD area (%) was determined. The tailing factor was computed using the chromatogram results.

For selectivity, the standard solution, placebo solution, mobile phase, and solvent were each injected 20 µL into the UHPLC system, and the chromatogram results were then compared. Linearity was determined by filling a 10 mL volumetric flask with exactly 10 mg of weighed ketoconazole. The flask was then filled with the methanol solution and swirled until fully dissolved, and the mixture was homogenised using ultrasound. The dilution obtained six different concentrations of the solution; each solution was filtered via a 0.45 µm membrane before being injected into the UHPLC apparatus. LOQ and LOD values obtained based on plotting the area of linearity test data against drug concentration, then determining the regression equation, slope, and intercept

Accuracy and precision were determined by preparing a 100% standard solution at concentrations of 15 µg/mL. 35 µg/mL. and 55 µg/mL. Each solution was injected three times consecutively. Recovery values percentage from the three injections of the standard solution must range from 98% to 102% for accuracy, and precision was achieved by the RSD (%) value from the three injections of the standard solution was $\leq$ 2.0%.[14.12.15.16].

A robustness procedure was conducted as in the precision test by altering the mobile phase composition

of acetonitrile: water 0.1% at pH 6, pH 5.5, and pH 6.5, followed by the determination of the concentration in the sample solution. The concentration and RSD area (%) from each sample injection time were calculated. The variations resulting from these changes were then compared[14].

Evaluation of Ketoconazole Niosomal Gel:

Evaluation of Ketoconazole Niosomal Gel includes organoleptic, pH value, viscosity, adhesion strength, and spreadability. All procedures were observed at room temperature according to standard pharmaceutical procedures. Organoleptic tests were performed on the prepared gel formulations, including observations of the color, odor, and physical appearance of the gel [17]. pH value of the gel was determined using a pH meter, and the value must meet the criteria for skin compatibility and non-irritation in the range of 4.5–6.5[18]. The viscosity was conducted using a viscometer (AntonPaar, Austria) on 250 mL of the gel formulation. The required viscosity for a good gel ranges from 2000 to 4000 mPas [49, 50]. An adhesion test was conducted by placing 0.5 grams of gel on a glass plate, then a 500 g weight was placed on top for three minutes, and covered with another plate. The bond strength was determined using the time it took to separate the two sheets of glass. This force must be greater than one second. [49, 51]. After adding 0.5 grams of gel to the middle of a measuring glass plate, a second glass plate is placed on top of it. The spreading test was assessed in terms of diameter, both horizontally and vertically, with 50-gram weights added each time until a total of 150 grams was reached. The spreadability that meets the criteria is in the range of 5-7 cm (18).

RESULT:

Optimization of Ketoconazole Niosomes:

Response Surface Methodology (RSM) is used to optimise techniques in the development of new processes and products, enhancing product quality and appearance while reducing production costs in industry[7, 13]. In niosome formation, key components include cholesterol and surfactant, the two elements used in Central Composite Design that were used in this study. The design produced 13 formulations, focusing on responses such as entrapment efficiency (EE%), polydispersity coefficient (PDI), zeta potential (ZP) and particle size (PS). (Table-1)

The results of the optimisation of the two factors with the desired response targets, analysed using the Design-Expert software, yielded a single formula recommended by the system. The optimisation design and result for the ketoconazole niosome formulation with the established constraints can be seen in Table 2 and Figure 1.

Table 1. Evaluation Results of Factors on Ketoconazole Niosome Responses

Run	X_1(mg)	X_2(mg)	Y_1(nm)	Y_2(mV)	Y_3(%)
1	1	8	540.8 ± 45.180	-39.50 ± 3.179	99.84 ± 0.030
2	0.1	8	481.9 ± 13.914	-26.58 ± 1.183	99.86 ± 0.002
3	1	4	570.9 ± 8.471	-53.12 ± 2.932	99.77 ± 0.020
4	0.55	6	288.7 ± 58.340	-26.52 ± 1.780	99.80 ± 0.005
5	0.55	6	370.9 ± 21.620	-30.35 ± 1.371	99.73 ± 0.002
6	0.55	8.882	353.4 ± 40.553	-37.10 ± 2.993	99.83 ± 0.020
7	0.55	6	264.5 ± 58.340	-40.79 ± 1.391	99.81 ± 0.006
8	1.186	6	466.0 ± 12.625	-58.67 ± 0.845	99.82 ± 0.006
9	0.086	6	403.4 ± 33.877	-37.19 ± 27.118	99.87 ± 0.030
10	0.55	6	387.4 ± 15.052	-40.89 ± 1.801	99.74 ± 0.020
11	0.55	3.171	470.9 ± 16.609	-38.03 ± 7.415	99.78 ± 0.020
12	0.1	4	442.9 ± 19.279	-32.02 ± 3.433	99.81 ± 0.008
13	0.55	6	469.1 ± 25.311	-45.24 ± 1.672	99.83 ± 0.002

Table 2. Results of The Statistical Analysis of The Central Composite Design with Responses

Results of the statistical analysis of the Central Composite Design with responses including PS, ZP, PDI, and EE%.					
Factors		**Y_1**	**Y_2**	**Y_3**	**Y_4**
X_1	Coefficient	34.43	-8.05	0.0714	-0.0163
	p – value	0.2734	0.0093	0.1374	0.2202
X_2	Coefficient	-19.66	2.55	-0.0714	0.0238
	p – value	0.5192	0.3338	0.1374	0.0902
X_1X_2	Coefficient	-17.27		0.1428	0.0050
	p – value	0.6859	-	0.0465	0.7792
X_1^2	Coefficient	60.92	-		0.0303
	p-value	0.0907		-	0.0531
X_2^2	Coefficient	49.64	-		0.0103
	p-value	0.1541		-	0.4568
Intercept		355.72	-38.92	0.5271	99.78
Sum of squares		51884.04	570.27	0.1630	0.0134

Mean of squares		10376.81	285.14	0.543	0.0027
F-value		1.55	5.67	3.55	2.28
p-value		0.2894	0.0226	0.0610	0.1563
Lack of Fit		0.4913	0.6861		0.9772

X_1 Lipid; X_2 HLB Surfactant; Y_1 Particle size(PS); Y_2 Zeta potensial(ZP) Y_3 Polydispersity Index (PDI); Y_4 Entrapment efficiency % (EE%).

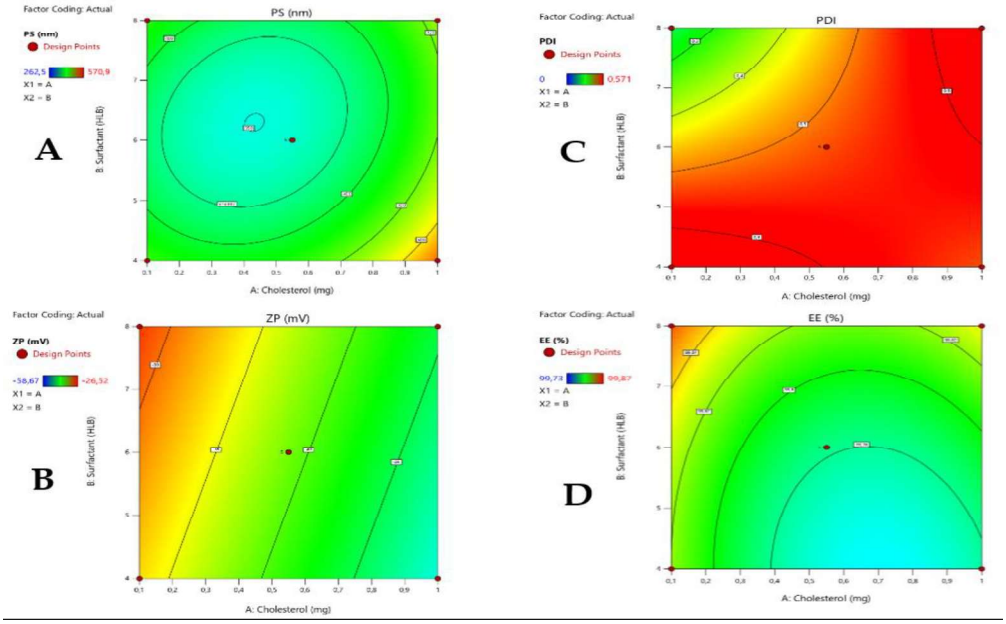

Figure 1. Two Dimensional Contour Plot of the Effect of Independent Variables Cholesterol – Surfactant HLB on the Response of Particle Size (A), Zeta Potential (B), Polydispersity Index(C), and Entrapment Efficiency% (D)

The results of the niosomes formula optimisation based on RSM can be seen in Tables 3 and 4 below. based on the findings in Table 4. Design-Expert software version 13 was used for formula optimization, with the parameters analyzed including particle size (PS), zeta potential (ZP), Polydispersity Index (PDI), and entrapment efficiency (EE%) were used to determine the optimal ketoconazole niosome. The optimal composition for the ketoconazole niosome included cholesterol at 0.1 mg and a surfactant HLB value of 7.294. with a desired value of 0.745. The ideal formulation obtained through the optimization procedure is the one that displays the highest desired value, with an optimization value approaching 1.

Table 3. Results of the Optimization of the Ketoconazole Niosome Formula

Name	Goal	Lower Limit	Upper Limit	Lower weight	Upper Lower	Impor tance
X_1	is in range	0.1	1	1	1	3
X_2	is in range	4	8	1	1	3
Y_1	Minimize	262.5	570.9	1	1	3
Y_2	Maximize	-58.68	-26.52	1	1	3
Y_3	Minimize	0	0.571	1	1	3
Y_4	Maximize	99.73	99.87	1	1	3

Table 4. Results of Ketoconazole Niosome Optimization Evaluation

Response	Predicted values	Actual value[1]	Error[2] (%)
Y1 (nm)	349.728	353.433 ± 81.941	1.059
Y2 (mV)	-36.440	-37.1 ± 7.094	-1.811
Y3	0.491	0.571 ± 0.123	16.29
Y4 (%)	99.792	99.786 ± 0.034	-0.006

[1]Data are presented as Mean ± standard deviation. n = 3;
[2]Error (%) = [(Actual value – Predicted value) / Predicted value] * 100%

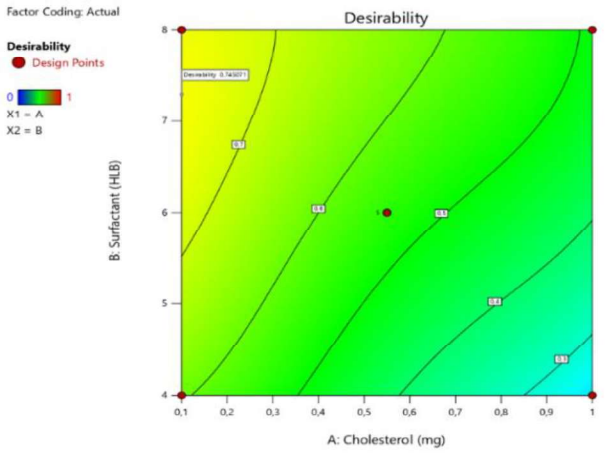

Figure 2. Contour plot of the desirability value of the optimal ketoconazole niosome formulation

Optimization of UHPLC System:

To improve the mobile phase, ensure the concentration of acetonitrile and air is adjusted to pH 6. The required amount is 20 µl, the concentration is 205 nm, and the amount is 1.0 ml/volume. The compatibility phase ends with the following explanation: (A) Acetonitrile: Water pH 6 (65:35), (B) Acetonitrile: Water pH 6 (60:40), and (C) Acetonitrile: Water pH 6 (55:45). All of which resulted in good peak shapes[12, 28]. The results of the optimisation and suitability tests are as shown in Tables 5 and 6 below.

Table 5. Results of the Mobile Phase Composition UHPLC Optimisation

Mobile Phase Acetonitrile: Water pH 6	Flow Rate (mL/min)	Area Under Curve (µV*sec)	Retention Time (minutes)	Theoretical Plate Count	Tail Factor
A (65:35)	1.0	890358 ± 23,094	2.455	5728.159	1.085299
B (60:40)	1.0	943879 ± 5,773	1.979	5395.062	1.133856
C (55:45)	1.0	910071 ± 20,663	2.467	5726.859	1.107983

Table 6. System Suitability Test Results

No.	Area Under Curve (µV*sec)	Retention Time (minutes)	Theoretical Plate Count	Tail Factor
1	1627980	2.029	5621.814	1.108
2	1627617	2.028	5609.588	1.110
3	1629246	2.027	5588.417	1.114
4	1627993	2.028	5616.890	1.109
5	1629972	2.028	5607.970	1.111
6	1628104	2.029	5615.454	1.109
Mean	1628485.425	2.028	5610.022	1.111
Stdev	914.874	0.001	10.699	0.002
%RSD	0.1	0.0	0.1	0.1
Syarat	%RSD ≤ 2.0%	%RSD ≤ 2.0%	≥ 2000	≤ 2.0%

Method validation results as shown in the table shown in the following Table 7 and 8 below:

Table 7. Results of Linearity, Accuracy and Precision Tests of The System

Linearity Test Results				
No.	**C Teoritis (µg/mL)**	**Area Under Curve (µV*sec)**		
1	10	762256		
2	20	1410511		
3	30	2226524		
4	40	3082806		
5	50	3720777		
6	60	4692984		
y = 77800x – 86400; r : 0.9993				
Accuracy Results				
No.	**C (µg/mL)**	**%RSD *Intra – day***	**% RSD *Inter – day***	**Criterion**
1	15	0.095 ± 0.07	0.462 ± 0.55	≤ 2%
2	35	0.070 ± 0.16	0.738 ± 0.66	
3	55	0.197 ± 0.12	0.195 ± 012	
Precision Results				
No.	**C (µg/mL)**	**%RSD *Intra – day***	**% RSD *Inter – day***	**Criterion**
1	15	0.095 ± 0.07	0.462 ± 0.55	≤ 2%
2	35	0.070 ± 0.16	0.738 ± 0.66	
3	55	0.197 ± 0.12	0.195 ± 0.12	

Table 8. Robustness Results

No.	C (microg/mL)	pH	Area Under Curve (µV*sec)	% RSD
1	15	5.5	1077442	0.195
	55		4223786	0.049
2	15	6.0	1101239	0.105
	55		4204866	0.202
3	15	6.5	1057147	0.382
	55		4167982	0.059

*noted: n = 3

Physical evaluation of niosome ketoconazole gel can be seen in Table 9:

Table 9. Physical evaluation Niosome Ketoconazole Gel

Formula			
Parameter	**F1**	**F2**	**F3**
Organoleptic	Semisolid, clear. Odorless	Semisolid, clear, odorless	Semisolid, clear, odorless
Homogeneity*	+	+	+
pH	5.5 ± 0.00	5.5 ± 0.00	5.5 ± 0.00
Viscosity (mPa.s)	3070 ± 34.9	3842 ± 13.95	4193 ± 14.71
Spreadability (cm)	6.6 ± 0.15	5.8 ± 0.7	6.2 ± 0.6
Adhesion (minute)	1.4 ± 0.14	1.5 ± 0.05	1.8 ± 0.07

Determination of Ketoconazole Niosomal gel Content

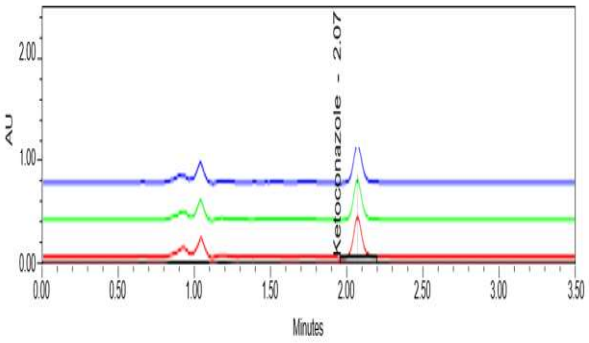

Figure 3. Overlay Ketooconazole in Niosomal gel

DISCUSSION:

The results of the optimisation of the two factors with the desired response targets, analysed using the Design-Expert software, yielded a single formula recommended by the system. The optimisation design for the ketoconazole niosome formulation with the established constraints can be seen in Table 2.

Based on the ANOVA results for Particle Size (PS) presented in Table 2. there is an interaction between the particle size. factors. and response. The equation derived from the statistical test is quadratic. where this equation links the response and variables as follows:

$Y_1 = + 355.72 + 34.43\ X_1 - 19.66X_2 - 17.27X_1X_2 + 60.92X_1^2 + 49.64X_2^{2:}$

Particle size is an important factor in enhancing the transdermal flux of ketoconazole. The particle size (PS) results which is range from 264.5 ± 58.3 nm to 540.8 ± 45.2 nm. The equation above shows that the particle size of ketoconazole niosomes, as observed for X1. indicates that an increase in cholesterol within the vesicle can affect the particle size. The relationship between the amount of cholesterol and the particle size of ketoconazole niosomes shows a direct correlation to the size of the vesicles as well as the amount of drug encapsulated within their bilayers. Previous studies have shown that increasing lipid content results in more drug molecules moving into the hydrophobic region of the vesicle, increasing the distance between the lipid bilayers and resulting in larger particle sizes[17.18.28.29.]

The equation for (X2) shows that the amount of surfactant does not significantly affect the negative results for the particle size (PS) of ketoconazole niosomes. This is probably due to the formation of micelles at high surfactant concentrations. which results in smaller particle sizes [19.20]. In this linear model, the effect is not significant because the p-value is≥ 0.05. The ANOVA results indicate that this variable does affect the particle size response, as the P value of 0.2894 is not significant (≥ 0.05).

Zeta potential (ZP) refers to the electrical charge present on the nanovesicles, it can be considered an indirect measurement of the stability of niosome dispersion[26.32]. In niosome formulations, a ZP value of ± 30 mV is considered stable[33]. In the results of this study's design, all ketoconazole niosome formulas produced ZP values ranging from -26.52 ± 1.78 mV to -58.67 ± 0.84 mV. Therefore, the ketoconazole vesicles possess sufficient charge to protect against aggregation and fusion. thereby maintaining their stability (34).

Figure 1 (B) shows the 3D response results for the effect of cholesterol amount (X1) and surfactant amount (X2) on ketoconazole niosomes. The results of the ANOVA indicate that the ZP of the niosome vesicles formed is significantly affected by one of the factors studied (P = 0.0226). where p ≤ 0.05. The model obtained is linear. with the equation that links the response and the variables as follows:

$Y_2 = -38.92 - 8.05\ X_1 + 2.55\ X_2$

The ketoconazole niosomes from the experimental design Table 1 resulted in a PDI range from 0.00 ± 0.0 to 0.571 ± 0.0. Figure 1 (C) shows the 3D response results for ketoconazole niosomes. illustrating the effect of cholesterol amount (X1) and surfactant amount (X2). The ANOVA test data for PDI indicates that the interaction model of the two factors (2FI) does not significantly affect the results. The equation derived is as follows:

$Y_3 = + 0.5271 + 0.0714\ X_1 - 0.0714\ X_2 + 0.1428\ X_1X_2$

ANOVA analysis result for EE% presented in Table 1 shows an interaction between the entrapment efficiency and the factors and response. The equation derived from the statistical analysis is quadratic, where this equation relates the response to the variables as follows:

$Y_4 = + 99.\ 78 - 0.0163\ X_1 + 0.0238X_2 + 0.0050X_1X_2 + 0.0303X_1^2 + 0.0103X_2^2$

The results suggest that the increased surfactant amount can cause the formation of leak points in the niosome vesicles[13]. This results in increased ketoconazole release and reduced ketoconazole storage[38]. In this quadratic model, the results are not significant, as the p-value > 0.05. The ANOVA results show that this variable affects the EE% response because the P-value is not significant at 0.6326 ≥ 0.05.

The two-dimensional (2D) visualisation results in Response Surface Methodology (RSM) are closely related to desirability values. In the context of RSM, the desirability function is used to optimise responses in an experiment by modifying input variables so that the response (dependent variable) reaches optimal conditions. Desirability values are calculated based on specific criteria, such as maximising or minimising the response variable outcomes[39]. Therefore, areas in the 2D plots that show high desirability indicate combinations of input variables that are close to the optimal response [26.27].

Based on the research, plots A, C, and D show contours with low desirability values. This suggests that, under those conditions, the combination of cholesterol and surfactant does not significantly influence particle size, Polydispersity Index, or entrapment efficiency. In contrast, plot B displays areas with high desirability values, indicating that certain combinations of surfactant and cholesterol significantly affect the Polydispersity Indexand approach optimal outcomes. Thus, this study demonstrates that the use of 2D RSM plots combined with desirability analysis is effective in optimising experiments and achieving the desired experimental outcomes[13 26.27].

The optimal composition for the ketoconazole niosome included cholesterol at 0.1 mg and a surfactant HLB value of 7.294, with a desired value of 0.745. The ideal

outcome of the optimisation process is the one that shows the maximum desired value, with an optimisation value approaching one. The required value range is from 0 to 1.

Figure 2 illustrates a two-dimensional surface where areas with low desirability values are located in the lower regions, while areas with high desirability values are found in the higher regions. approaching a value of 1. In this study, the response values displayed in Table 5 were anticipated by the software. These predictions were validated through three confirmatory tests to ensure optimisation, and estimates were validated by observations of the optimised ketoconazole niosomes, which showed acceptable variations compared to the predicted values.

Mobile phase optimization was performed using acetonitrile and water at pH 6, in various proportions. The compositions of the mobile phase were as follows: (A) Acetonitrile: Water pH 6 (65:35), (B) Acetonitrile: Water pH 6 (60:40), and (C) Acetonitrile: Water pH 6 (55:45). All of which resulted in good peak shapes[12, 28].

From the optimisation results at a flow rate of 1.0 mL/min, the retention time for ketoconazole with mobile phase A was 2.455 minutes, with mobile phase B was 1.979 minutes, and with mobile phase C was 2.467 minutes. However, the chromatogram obtained with mobile phase B showed the largest area and the best chromatogram shape. Based on this optimisation, the highest theoretical plate count and column capacity. as well as the system suitability test parameters. still met the required criteria for this mobile phase variation. Consequently. Acetonitrile was the ideal mobile phase composition for the analysis. pH 6 water (60:40).

The System Suitability Test (SST) is performed to ensure that the UHPLC system operates with optimal parameters, which generally include theoretical plate count (column efficiency), capacity factor, resolution, tailing factor (peak symmetry), and the relative standard deviation (%RSD) of the peak surface and retention time following several successive injections41. Parameters such as the theoretical plate count (>2000) and tailing factor (≤2) are commonly used as reference standards in SST. These parameters are intended to assess column efficiency. peak symmetry. and the column's ability to separate the components. In this research, the system performance test (SST) was performed using six successive administrations. During this phase, metrics such as the relative standard deviation (RSD) of retention time and peak area are assessed to verify that the system produces consistent results (43). The results of the SST in this study for each parameter met the required specifications, as shown in Table 9. This result showed that system ready and suitable for further analysis.

The linearity test results with a correlation coefficient (r) of 0.9993 indicate that the validated method has a very strong linear relationship between the analyte concentration and the instrument response. This value exceeds the minimum threshold recommended in method validation guidelines. where values above r = 0.999 are considered sufficient to demonstrate good linearity [21].

After performing the selectivity test, the chromatogram of the ketoconazole compound was obtained with a retention time of 2.096 minutes. A comparison test was then conducted between the chromatograms obtained using the mobile phase, solvent, gel base, and niosome-forming materials. The results showed that no other compound provided a response at the same time as the retention time of ketoconazole, with only the ketoconazole standard producing a peak area. Therefore, the selectivity test for the validation of the ketoconazole determination analysis method meets the criteria.

In technical validation, two crucial metrics are the limit of quantification (LOQ) and the limit of detection (LOD), particularly to ensure the sensitivity of a method and its ability to detect and measure the analyte at low concentrations[22.23]. These two thresholds are determined based on the response deviation and the slope of the regression graph established by the linearity test. From the ketoconazole linear regression curve, the regression equation y = 77.800x – 86.400 (µg/mL) was obtained, and the LOD for ketoconazole was calculated as 2.4315 µg/mL, and the LOQ was 7.3683 µg/mL. The lower LOQ value indicates that the proposed method is suitable for analyzing samples containing ketoconazole, even in small amounts.

Accuracy testing was conducted both intra-day and inter-day. The testing involved analyzing three concentrations (low, medium, and high) with three replicates for each concentration. The intra-day accuracy test at the three concentrations yielded %. Recovery numbers, which are displayed in Table 8, demonstrate that the outcomes satisfied the accuracy acceptance standards, which is 98–102% [21]. The inter-day accuracy test at the three concentrations was performed over three days, and the % recovery results in Table 7 met the acceptance criteria of 98–102%. Thus, the method continues to yield consistent and accurate results over different days.

The results of the precision test, both Intra-day and inter-day, in this study Table 7 met the precision acceptance criteria. with % RSD ≤ 2% [21]. These results

can be concluded to demonstrate that the method used has good consistency and accuracy. This indicates that the measurements are able to produce stable and consistent data both under the same conditions (intra-day) and different conditions (inter-day). A measurement method is considered robust if it demonstrates consistency in use, even in the face of minor and deliberate changes in its parameters. Depending on the procedure in question, this robustness assessment is essential during the method development stage. The purpose is to ensure that the analytical method remains stable even when deliberate variations occur in specific parameters. One such influence is the variation in pH within a mobile phase [24].

In this work, the robustnest test was carried out by varying the pH of the mobile phase between 5.5, 6.0, and 6.5. The results shown in Table 8 were obtained from three concentrations, and the results indicated that these changes did not affect the performance of the method, with % RSD values ranging from 0.049 to 0.38, suggests that the technique validation is appropriate for measuring ketoconazole concentration in samples.

The preparation of the gel base using Carbopol 940 as a gelling agent, with three different concentrations. The variation in Carbopol concentration as the gel-forming agent affects the thickness or viscosity of the formulation. Therefore, the consistency of the resulting gel also varies[25]. Besides the gelling agent, the preparation of a gel formulation also requires additional ingredients. In the process of adding water or other additives in the form of a solution to Carbopol, the volume of the mixture will increase, but the gel will still maintain its consistency[25.27]. Furthermore, water plays a role in facilitating the mixing of active or other additional components in the gel, allowing for an even distribution, which is important in topical or cosmetic applications [29].

Each formula's organoleptic test findings demonstrated that the gel was semisolid, odourless, and transparent, and showed was homogeneous without any visible powder particles Table 9. This indicates that the ketoconazole niosomes met the homogeneity standard. Homogeneity is important to ensure that the active ingredient is evenly distributed so that each dose or application contains a consistent amount of the active ingredient.

The pH test results for the three formulas ketoconazole gel showed the same pH of 5.5 ± 0.0. This indicated that the formulation meets the requirements for topical preparations with a pH compatible with the skin, which is between 4.5 and 6.5[30]. According to previous studies, gel formulations with an acidic pH of around 5.5 support optimal skin function, while a pH that is too alkaline can disrupt the skin's lipid layer and increase dryness and the risk of infection [31].

The viscosity test results shown in Table 9 indicate that all three gel formulas have viscosities that meet the acceptable criteria, ranging from 2000 to 4000 mPa.s [42, 43]. This viscosity reflects the thickness of the resulting formulation. Carbopol 940 at higher concentrations results in formulations with higher viscosity. The spreadability test results in this study also meet the requirements, falling within the range of 5 – 7 cm [32.33.25]. A formulation that spreads well ensures an even distribution over the application area. enhancing therapeutic effectiveness or protection. The adhesion test results in this study, as shown in Table 9 indicate that the formula meets the requirements with a duration of more than 60 seconds.

After optimising the gel formulation using Carbopol 940 at three different concentrations, all of the formulas met the required physical characteristics for ketoconazole niosomal gel. Based on these results, the formulation with the lowest Carbopol concentration, F1 (0.5%), was selected for further study. This selection was based on the efficient use of materials and satisfactory physical evaluation results.

The chromatographic profiles of non-niosomal ketoconazole gel, commercial ketoconazole cream, and niosomal ketoconazole gel showed a major peak at a similar retention time of approximately 2.07 minutes, confirming the presence of ketoconazole in all formulations[34]. The consistent retention time across different dosage forms indicates good selectivity of the analytical method. The assay results were within the theoretical range (98–102%), demonstrating accurate quantification of ketoconazole without interference from the niosomal gel components. Furthermore, the overlay chromatogram of ketoconazole niosomes Figure 3 showed overlapping peaks among all formulations, indicating uniformity and purity of the active substance

CONCLUSION:

The study demonstrates that the UHPLC technique was validated to quantify ketoconazole in Niosomal gel formula. The characteristics important, like selectivity, linearity, accuracy, precision, and LOD, were used to ensure the validation process meets the criteria.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this research.

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