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Development and Validation by UV Spectrophotometric method for Simultaneous Estimation of Fluconazole and Thymol in Formulation

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Abstract: ***Simultaneous equation technique has devised a simple, rapid, accurate, precise, and cost-effective spectrophotometric strategy for simultaneous estimation of Fluconazole and Thymol. Fluconazole and Thymol had absorbance maxima at 261 and 273 nm, respectively; hence absorbance was measured at these wavelengths for fluconazole and Thymol estimate. Fluconazole and Thymol followed Beer-Lambert's rule in concentration ranges of 20-140 g/ml and 20-100 g/ml, respectively. The technique was developed and verified in compliance with ICH guidelines and may be used to estimate Fluconazole and Thymol concentrations within formulations.***
Keywords: Fluconazole, Thymol, Simultaneous equation, U.V. Spectrophotometer, Validation.

I. INTRODUCTION

Spectrometry is concerned with equipment that detects or emits electromagnetic radiation as a result of its interaction with materials. The measurement of electromagnetic radiation absorbed by atoms, molecules, or ions of certain wavelengths is known as absorption spectrometry [1].

The quantity of absorption is determined by the wavelength of the light and the compound's structure. Radiation absorption occurs when electrons in lower energy orbitals are stimulated into higher energy orbitals. Because this is an electron transition phenomenon, UV is sometimes referred to as electronic spectroscopy [2, 3]. UV visible spectrophotometry is a technology that is commonly used in pharmaceutical analysis. It entails measuring the quantity of ultraviolet (190-380nm) or visible (380800nm) radiation absorbed by a material in solution using equipment that measures the ratio or a function of the ratio of the intensity of two light beams in the UVvisible area [4].

Fluconazole is an antifungal medicine that is a synthetic triazole derivative with the chemical formula 2-(2, 4-difluorophenyl)-1, 3-bis (1H-1, 2, 4-triazol-1-yl).-2-propanol (Fig 1). It is effective against a wide range of systemic and superficial fungal diseases [5]. Fluconazole is a BCS Class II medication with strong permeability and low solubility. Hence it is insoluble in water and highly soluble in organic solvents. Fluconazole interacts with the 14-demethylase cytochrome P-450 enzyme, which catalyses the conversion of lanosterol to ergosterol. It inhibits the synthesis of ergosterol, which is required for fungal cell membrane development, and increases cellular permeability [6]. Thymol, also known as 2-isopropyl-5-methylphenol, (Fig 2) is a monoterpene phenol that is prevalent in plants such as *Thymus vulgaris.* Due to the deprotonation of phenol, it is highly soluble in alcohols, alkaline solutions, and other organic solvents, but it is only slightly soluble in water at neutral pH and absorbs the most UV radiation at 273 nm.

Thymol disrupts cell wall or membrane integrity and interferes with ergosterol biosynthesis [7]. To treat fungal infections caused by Candida albicans, a combination of fluconazole and thymol has been developed. Since synthetic drugs are resistant to Candida albicans, combining them with natural constituents produces synergistic efficacy. The literature search revealed that, although a promising combination, there is no method available for simultaneous estimation of thymol and fluconazole from dosage form. Therefore, developing a UV spectrophotometric method for the simultaneous estimation of thymol and fluconazole is challenging. Therefore, the aim of the present work was to develop & validate a UV spectroscopic method for simultaneous estimation of Fluconazole and thymol from Spray formulation.

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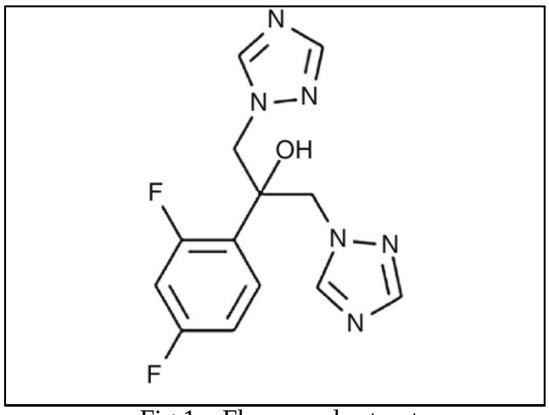

Fig 1 – Fluconazole structure

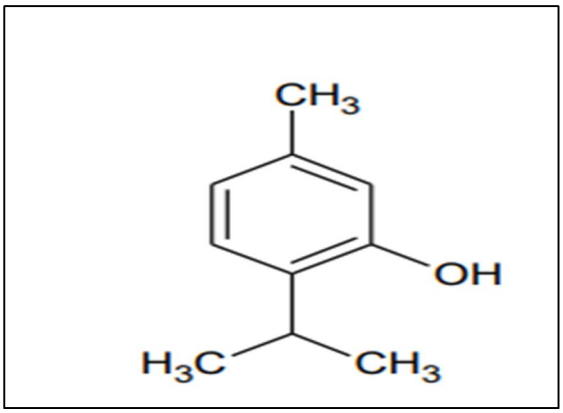

Fig 2 –Thymol Structure

II. MATERIALS AND METHODS

A. Chemical and instrument

The standard drug Fluconazole was received as gift sample from FDC (Mumbai) and Thymol from Powder Pack Chem India. Ethanol was purchased from Vishal chem. Kolidone VA64 polymer was obtained from Ashland India Pvt Ltd. Ethyl Cellulose was obtained from Vishal chemicals. All other excipients were procured from vishal chemicals. All solvents and reagent used were of analytical grade. Over the range of 200-400 nm, a UV-spectrophotometer UV-1800 (Shimadzu, Japan) with a spectral bandwidth of 2 nm and 10 mm matched quartz cells was utilised to create an analytical procedure.

B. Method

1) Method of Preparation of film forming spray

- Fluconazole and thymol was separately dissolved in a vehicle mix of 1: 1 Ethanol and Diethylene glycol monoethyl ether.
- Polymer solution was prepared containing plasticizer, preservation and pH Balancer, and gradually added to fluconazole and thymol solution.
- The solution was stirred for 15 min at 80–100 rpm before being sonicated for 10 min.
- The solution was then placed in a refillable container with a 2 mm internal diameter plastic dip tube.

2) Selection of wavelength for analysis of Fluconazole and Thymol:

Fluconazole (100 mg) and Thymol (100 mg) were accurately weighed and transferred to a 100 mL volumetric flask, dissolved, and diluted to the mark with ethanol to generate a standard solution with Fluconazole and Thymol concentrations of 1000 ppm each. The concentration of the drugs 100µg/ml each in ethanol was generated from this stock solution and scanned using a spectrophotometer within the wavelength range of 200 to 800nm against ethanol as a blank(fig 3).

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3) Calibration of Curve of Fluconazole and Thymol

A series of calibrated 10mL volumetric flasks were taken and appropriate aliquots of stock solution of Thymol and fluconazole were withdrawn and diluted up to 10mL with water, Different dilutions were made from this stock solution, ranging from 20µg/ml to 140µg/ml for Fluconazole. The absorbance was measured at absorption maxima 261 nm for Fluconazole against the reagent blank prepared in a similar manner without Fluconazole with Ethanol (Fig4). The same procedure was applied for Thymol stock solution solutions having concentrations of 20µg/mL to 100µg/mL and absorbance was measured at 273nm, against a reagent blank prepared in a similar manner without Thymol with Ethanol (fig 5).

4) Simultaneous Equation Method [8-10]:

If a sample contains two absorbing drugs, each of which absorbs at the λ_{max} of the other, it may be possible to determine both drugs simultaneously using multicomponent analysis UV Spectrophotometric 'Simultaneous Equation Method. Two simultaneous equations (in two variables C_x and C_y) were formed using these Absorptivity coefficient values. Concentrations in the sample were obtained by using the following equations.

$$C_X = A_2ay_1 - A_1ay_2 / ax_1ay_2 - ax_2ay_1 \ldots\ldots (1)$$

$$C_y = A_1ax_1 - A_2ax_1 / ax_1ay_2 - ax_2ay_1 \ldots\ldots (2)$$

Where, A1 and A2 are absorbance's of the Sample at 261 nm and 273 nm respectively, ax1 and ax2 are absorptivities of Fluconazole at 2 wavelengths respectively and ay1 and ay2 are absorptivity of Thymol at 2 wavelengths respectively. Cx and Cy are concentrations of Fluconazole and Thymol respectively.

C. Validation of the Method [11-16]

The UV-VIS Spectrophotometric technique was verified in accordance with the International Conference on Harmonisation (ICH) requirements. For validation, the following qualities were taken into account: linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ).

1) Linearity

An analytical procedure's ability to be linear is its ability to rationally demonstrate that the observed absorbance is proportionate to the concentration of a sample containing the analyte. Fluconazole's linearity was investigated at 261 nm using seven different concentrations that ranged from 20 - 140 µg/mL. Thymol's linearity was also investigated using five different concentrations that ranged from 20 - 100 µg/mL. Plotting concentration vs absorbance allowed for the creation of the calibration curves. Fitting to the equation y = mx + c yielded the regression's slope, intercept, and correlation coefficient values (Table 1).

2) Precision

Precision is defined as the closeness of the readings obtained by multiple measurements of the same sample under prescribed conditions (Table 3 & Table 4). Intraday and interday precision are considered for the precision studies· Intraday Precision: The absorbance of fluconazole and thymol solution samples at concentrations of 20, 60, and 100 g/mL was acquired three times on the same day and the % RSD was determined. Interday Precision: The absorbance of Fluconazole and Thymol sample solutions at concentrations of 20, 60, and 100 g/mL was tested on three different days and the percentage RSD was calculated.

3) Accuracy

The accuracy of an analytical approach displays the degree of agreement between the values considered as conventional true values and the value discovered. The method's accuracy was established by conducting recovery experiments. The recovery investigations were carried out by mixing the previously analysed formulation sample solution with the reference medication solution. The data had to be accurate within 2% standard deviation (SD) of nominal values and precise within 2% relative standard deviation (RSD) to be accepted (Table 5).

4) Limit of Detection (L.O.D)

The lowest concentration of analyte in a sample that can be detected but not always quantitated as an accurate number is referred to as the detection limit of an analytical procedure. The standard curve was used to determine the limit of detection.

$$L.O.D = \frac{3.3 * N}{S}$$

Where N is the standard deviation of the absorbance of the sample and S is the Slope of the calibration curve (Table 1).

5) Limit of Quantification (LOQ)

The limit of quantification (LOQ) is the smallest quantity of analyte that can be quantitatively measured in a sample with sufficient precision and accuracy (Table -1). The limit of quantification was calculated using the following equation as the response's standard deviation and the slope of the associated curve:

$$L.O.Q - \frac{10 * N}{S}$$

Where N is the standard deviation of the absorbance of the sample and S is the Slope of the calibration curve.

6) .Molar absorptivity

Molar absorptivity (ε) it is a measurement of how strongly a chemical species absorb light at a given wavelength. It is an intrinsic property of the species (Table 6) The actual absorbance (A) of a sample is dependent on the path length (l) and the concentration (c) and is given as-

[$A = \varepsilon cl$]

The unit used to describe the molar absorptivity is L/mol/cm.

Calculation of molar absorptivity: -

Molar absorptivity (ε) = Absorbance/molar concentration

Calculation of percent absorptivity: -

% Absorptivity = Absorbance/concentration in mg/ml

7) Assay

The proximity of the measured value to the real value is referred to as accuracy. The recovery experiments were used to assess the method's accuracy. The recovery investigations were conducted by spiking the previously analysed formulation sample solution with the standard medication solution.

8) Ruggedness

The ability of an analytical technique to produce appropriate findings with minor, intentional changes to the method parameters is known as robustness (Table 8).

III. RESULTS AND DISCUSSION

A. Absorption Maxima

In the resulting spectra, compounds such as fluconazole and Thymol display their maximum absorption peak at 261nm and 273 nm in the wavelength range of 200 to 800 nm versus ethanol as a blank. Overlain spectra of the drugs revealed the presence of two peaks at 261nm and 273nm, as seen in the fig 3.

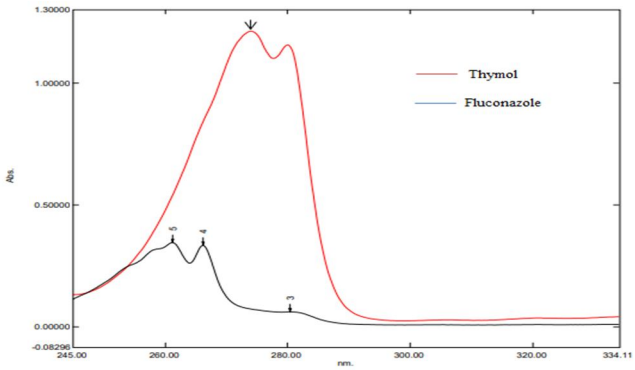


Fig 3 – Overlay spectrum of Fluconazole and Thymol

B. Linearity and Range

Linearity range for Fluconazole and Thymol are 20-140 µg/ml and 20-100 µg/ml at selected wavelengths respectively (Table-1). The coefficient of correlation for fluconazole at 261 nm and for Thymol at 261 nm is 0.9998 and 0.9996 respectively. Both drugs shows good regression values at their respective wavelengths [Fig no – 4, 5]

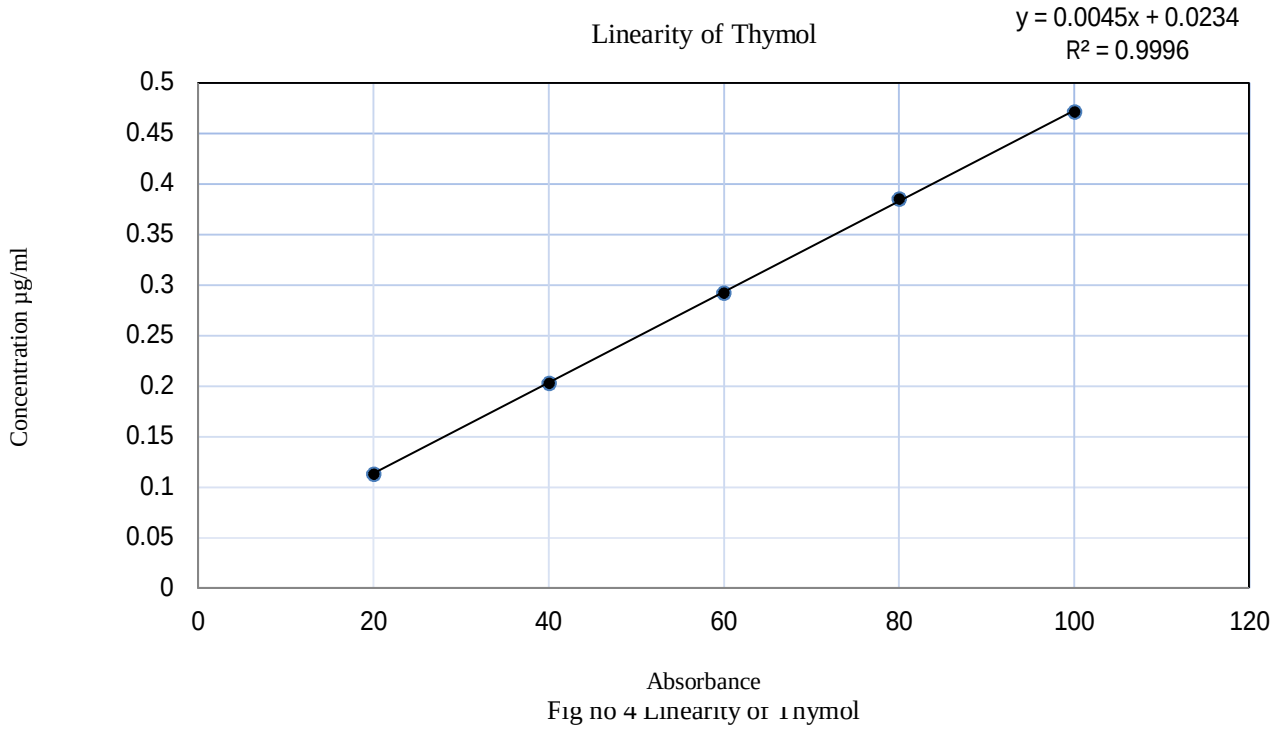
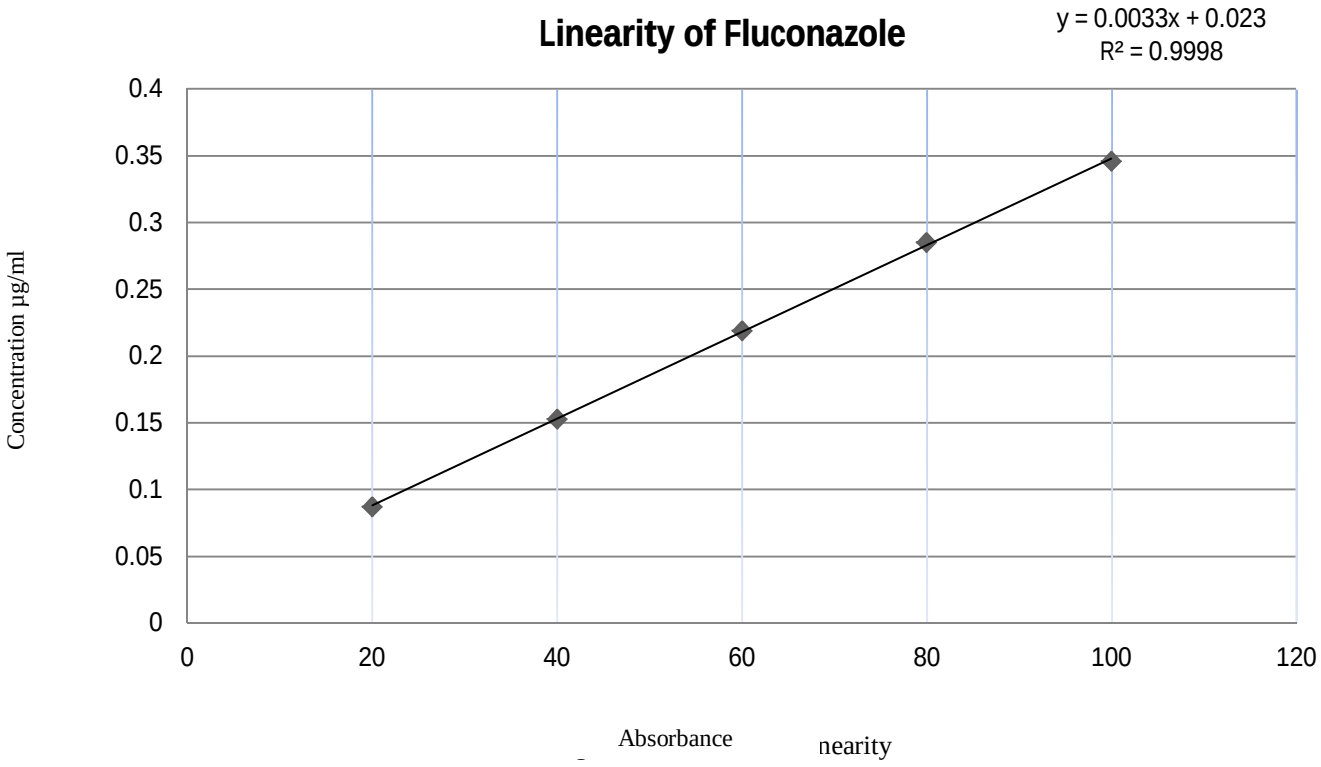


Fig no 4 Linearity of Thymol



nearity

Table 1: Result of validation parameter

Parameters	Fluconazole	Thymol
λmax	261nm	273nm
Linearity	20-140 µg/mL	20-100 µg/mL
Regression equation	Y = 0.0033x + 0.023	y = 0.0045x + 0.0234
Standard Deviation	0.00430	0.0039
%RSD	0.293	0.335
Correlation coefficient	0.9998	0.9996
LOD	4.30	2.87
LOQ	13.05	8.72

C. Precision

The repeatability (inter-day) and intermediate precision (intra-day) precision studies of the developed method confirmed that the method is precise and reliable where all the RSD values were <2%.

1) Repeatability

The % RSD value was found to be less than 2% indicating this method is precise for the determination of both the drugs. (Table- 2)

Table 2 - Repeatability of Fluconazole and Thymol

Drug	Concentration	Absorbance	S.D.	% RDS
Fluconazole	40 ppm	0.151	0.00136	0.27066
Thymol	40 ppm	0.202	0.00041	0.20217

2) Intermediate Precision

Intermediate precision was performed by measuring the absorbance of the sample solution on three different days and on the same day (Table 3).

Table -3 Intermediate Precision

Theoretical Concentration (µg/mL)	Fluconazole			Thymol		
	Measured Absorbance	Standard Deviation	% RSD	Measured Absorbance	Standard Deviation	% RSD
20	0.081	0.001155	1.413919	0.111	0.001528	1.367933
60	0.214	0.004041	1.882664	0.287	0.003215	1.117457
100	0.343	0.006351	1.849763	0.466	0.005033	1.079319

3) Interday precision

Interday precision was performed by measuring the absorbance of the sample solution on three different days and on the same day (Table -4).

Table – 4 Interday Precision

Theoretical Concentration (µg/mL)	Fluconazole			Thymol		
	Measured Absorbance	Standard Deviation	% RSD	Measured Absorbance	Standard Deviation	% RSD
20	0.089667	0.001155	1.28777	0.114	0.002	1.754386
60	0.217	0.002646	1.21924	0.289333	0.004041	1.396815
100	0.346333	0.004726	1.364528	0.471667	0.004726	1.00194

D. Accuracy

The recovery trials yielded results ranging from 80 to 120% (98.23 to 106.2%), demonstrating that the technique is accurate and that commonly used excipients and additives in pharmaceutical formulations did not interfere with the recommended procedure. Recovery trials confirmed the method's accuracy, which was found to be significant and within specification limits. (i.e. within the recommended 98-120% recovery range (Table -5).

Table -5 Results of Accuracy

Drug	Concentration of Drug added		% Mean Recovery	Standard Deviation	% Recovery ±SD
	µg/mL	%Level			
Fluconazole	48	80	97.64	0.9643	0.9876
	60	100	100.58	0.3644	0.3623
	72	120	101.32	1.0934	1.0791
Thymol	48	80	97.96	1.2248	1.2503
	60	100	100.12	1.3364	1.3348
	72	120	103.75	1.8710	1.8034

E. Molar absorptivity (ε)

Molar absorptivity (ε) of Fluconazole and Thymol was calculated at 261 nm and 273 nm (Table 6)

Table-6 – Molar absorptivity of Fluconazole & Thymol

Wavelength (nm)	Molar absorptivity (ε) of Fluconazole	Molar absorptivity (ε)of Thymol
261	0.02396	0.00360
273	0.00132	0.09324

F. Application of Developed Method to spray formulation (Assay)

20 ml of the formulation was transferred to 100ml Volumetric as each 10 ml represent 100mg of per drug in the formulation, and filtered through Whatmann filter paper. Required concentrations 60 µg/ml and 40 µg/ml both the drugs were made by making necessary dilutions with Ethanol(Table -7).

Table -7 – Assay of Developed Spray formulation

Drugs	Label claim (mg)	Concentration found (mg)
Fluconazole	150	99.4
Thymol	150	98.8

G. Ruggedness

The ruggedness of developed method was checked by analysing Fluconazole & Thymol by different analysts at similar operational and environmental conditions. The % RSD values were found to be less than 2. (Table - 8).

Table - 8 Ruggedness of fluconazole & Thymol

Drugs	Analyst	Concentration µg/ml	Mean Absorbance	SD	% RSD
Fluconazole	Analyst 1	40	0.317	0.00041	0.12845
	Analyst 2		0.318		
Thymol	Analyst 1	40	0.279	0.06324	0.2258
	Analyst 2		0.281		

H. Robustness

Robustness of the proposed method was determined by estimating a drug at slightly different wavelength from the selected wavelength. No significant difference was found in the absorbance of samples. Therefore, the proposed method was considered as robust.

I. Specificity

The proposed method was found to be specific as there is no interference with other excipients.

J. Stability

The stability of the standard and sample solutions was tested at room temperature for three days, and the absorbance was determined on each day. The amount of medication contained in the sample solution was determined, and the findings verified that the sample solution is stable at room temperature for three days without deterioration.

IV. CONCLUSION

The proposed method, which uses a spectrophotometric method of analysis that is less expensive and simpler than methods like chromatography and electrophoresis, can be used for routine quality control analysis of Fluconazole and Thymol pharmaceutical dosage forms. The developed method was found to be sensitive, accurate, precise, reproducible, and linear over the concentration range studied.

V. ACKNOWLEDGEMENT

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VI. CONFLICT OF INTEREST

The authors report no conflicts of interest

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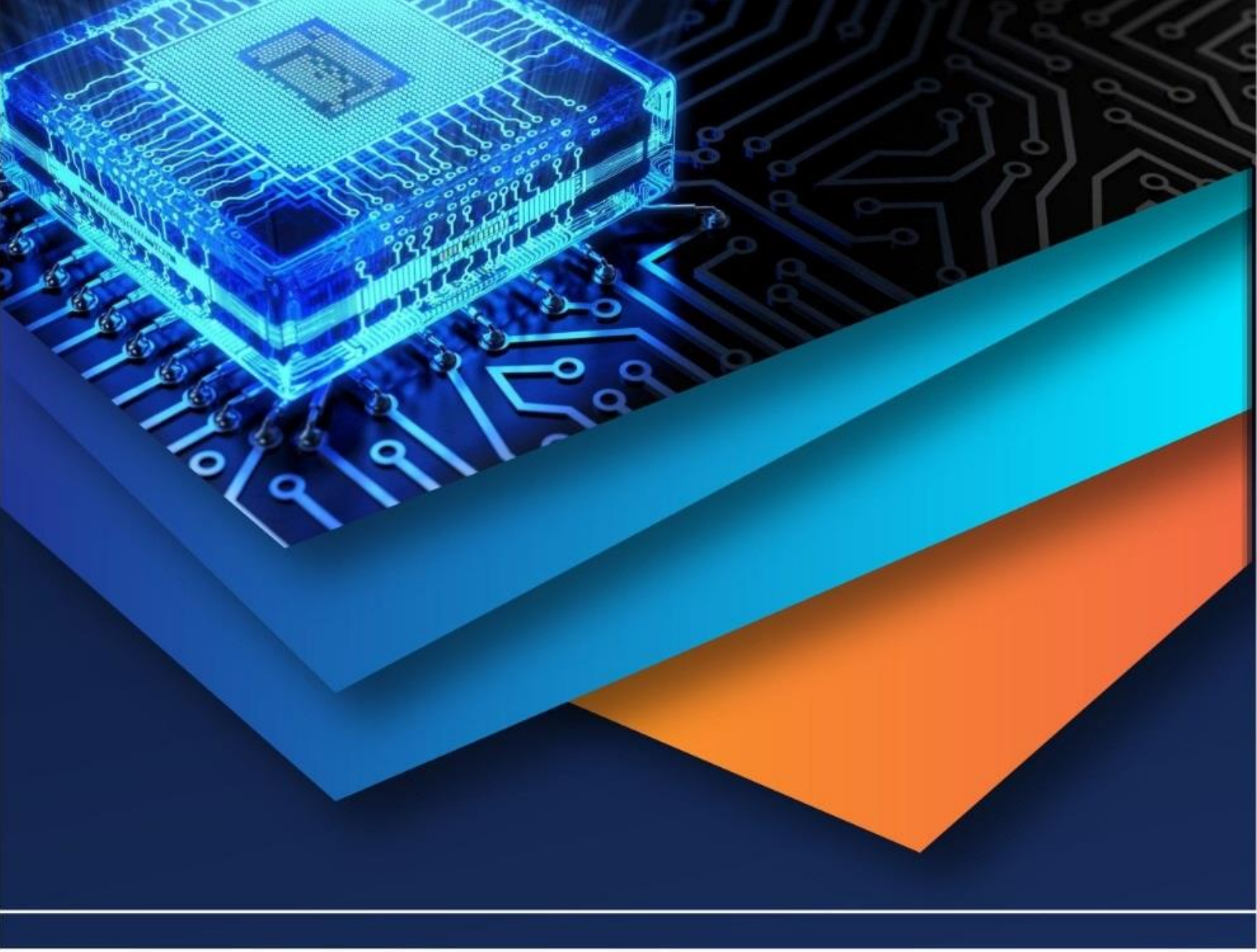

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