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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION STUDIES FOR SIMULTANEOUS ESTIMATION OF LULICONAZOLE AND EUGENOL USING UV SPECTROSCOPY

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Luliconazole, Eugenol, UV spectroscopy, ICH Q2(R1), simultaneous method

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ABSTRACT: A simple, precise yet novel UV spectrophotometric method has been developed for the simultaneous estimation of luliconazole and eugenol. Two methods, namely the simultaneous equation method and the absorbance ratio method were tried for simultaneous analysis. Since the simultaneous equation method gave the best recovery; the simultaneous method was developed for the estimation of both drugs. The wavelength of interest is the wavelength maxima of both drugs; λmax of luliconazole 296 nm and λmax of eugenol 282 nm. The correlation coefficient value (r^2) was found to be 0.99 for linearity studies both in methanol and phosphate buffer. Validation studies of the developed method were conducted as per ICH guidelines. The method was precise since the percent relative standard deviation was less than 2 and accurate since the percent recovery was between 99.42% and 105.16%. The developed method can be further used for simultaneous analysis of the drugs in bulk and formulation by UV spectroscopy. The development of a suitable analytical method for quantitative estimation is essential for assay and release studies.

INTRODUCTION: Fungal infections are on the rise and emerging to be a global problem with increased incidence of resistance to clinically used antifungal drugs. Fungi causing major fungal infections include *Candida spp., Aspergillus spp., Cryptococcus spp., Fusarium* spp., and *Trichophyton spp.* [1]. Luliconazole is one of the widely used drugs in the treatment of topical fungal infections and is available in various formulation types like cream, gel, lotion, powder, and soaps.

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It belongs to the azole category of antifungal drugs and was first discovered in Japan and [2]. It is chemically (2E)-2- [(4R)-4-(2,4-chlorophenyl)-1,3-dithiolan-2-ylidene]-2-imidazol-1-ylacetonitrile has a molecular formula of $C_{14}H_9C_{l2}N_3S_2$ **Fig. 1** and molecular weight of 354.28 g/mol [3, 4]. Luliconazole is available as a very fine yellowish powder, with a chiral center, and the R-isomer is used in formulations [5].

It is a broad-spectrum topical antifungal drug approved by the US FDA in November 2013 and launched in India by Ranbaxy Laboratories Ltd, however, it is not yet officially listed in IP, BP, and USP. It acts by blocking the enzyme lanosterol demethylase eventually resulting in inhibition of the ergosterol synthesis pathway [6]. Luliconazole 1% cream is used in topical fungal infections [7–10].

Eugenol is the major phytoconstituent of clove oil; chemically it is 2-methoxy-4-(2-propenyl) phenol that has a molecular weight of 164.20 g/mol and its molecular formula is $C_{10}H_{12}O_2$ **Fig. 1**. It is reported to be antioxidant, anti-inflammatory, anesthetic, antiviral, and antimicrobial. In fungi, it inhibits the cell wall by inhibiting ergosterol synthesis [11–14]. Our lab is working on the development of a topical hydrogel delivery system consisting of luliconazole and eugenol in combination (0.1 percent each) to achieve synergistic antifungal activity. The development of a suitable analytical method for quantitative estimation is essential for assay and release studies. This paper describes the analytical method development and validation studies conducted to establish the stability of the analytical method for assay and release studies of luliconazole and eugenol in developed formulations.

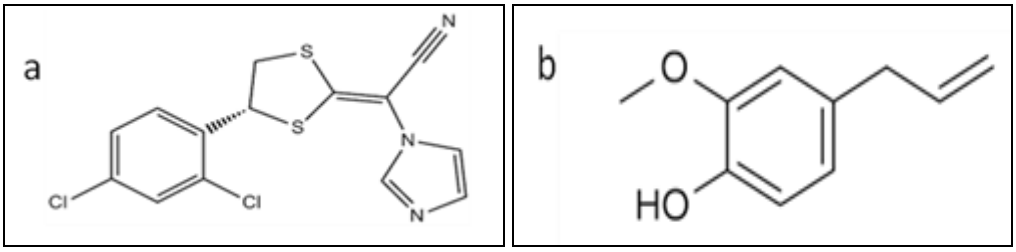

FIG. 1: MOLECULAR STRUCTURE OF (A) LULICONAZOLE AND (B) EUGENOL

MATERIAL AND METHODS: Luliconazole was obtained as a gift sample from Hema Pharmaceuticals Pvt. Ltd., Eugenol was procured from Tokyo Chemical Industry Co. Ltd., and analytical grade methanol was procured from SD Fine Chemical Ltd. All other solvents and chemicals used in this study were of analytical grade.

UV-Spectroscopy Method Development:

Selection of Detection Wavelength: In the present study, a 10 µg/mL solution of LZL and EUG each in methanol was prepared. These solutions were scanned between 200 - 400 nm and spectra were recorded. The wavelength at which both drugs showed maximum absorbance is reported [15–18].

Instrumentation: A double-beam UV-visible spectrophotometer, model- Shimadzu 1900, with a spectral bandwidth of 1.5 nm and automatic wavelength corrections with a pair of 10 mm quartz cells was used for the experimental work. Preparation of standard stock solution (1000 µg mL^{-1}). An accurately weighed quantity of 10 mg LZL and EUG each was transferred into a 10 mL volumetric flask and volume made up with methanol. The solution was sonicated for 10 min to obtain 1000 µg mL-1 stock solutions of LZL and EUG each. The absorption spectra were determined by scanning a solution, at 200-400 nm. The λmax for LZL and EUG was found to be 296 nm and 282 nm respectively. Preparation of a working solution for the calibration curve 1 ml stock solution (LZL & EUG) each was taken in a 10mL volumetric flask and volume was made up to 10 mL with methanol to obtain 100 µg mL^{-1} solution. From the standard LZL stock solution, 0.5, 1, 1.5, 2-, 2.5-, and 3-ml aliquots were taken in 10 mL volumetric flasks and made up to volume with methanol to obtain working solutions ranging from 5-30 µg mL^{-1}. From the standard EUG stock solution, 0.5, 1, 1.5, 2, 2.5, 3-, 3.5-, and 4-ml aliquots were taken in 10 mL volumetric flasks and volumes made up with methanol to obtain working solutions ranging from 5-40 µg mL^{-1}.

Method Development for Simultaneous Estimation of Luliconazole and Eugenol in Phosphate buffer pH 5.5:

Simultaneous Equation Method: The simultaneous equation method is based on the absorption of drugs LZL and EUG at their wavelength maxima in phosphate buffer. The λmax of LZL and EUG in phosphate buffer 5.5 was found to be 299 and 280 nm, respectively. The absorptivity values for LZL are 0.0414 (ax1), 0.0299 (ax2), and for EUG are 0.0012 (ay1), and 0.0184 (ay2) at 299 and 280, respectively. The absorbances and absorptivity of these drugs were substituted in equations (1) and (2) to obtain the concentration of drugs.

$$Cx = [A_2 a y_1] - [A_1 a y_2] / [a x_2 a y_1] - [a x_1 a y_2] \cdots\cdots\cdots (1)$$

$$Cy = [A_1 a_{x2}] - [A_2 a_{x1}] / [a_{x2} a_{y1}] - [a_{x1} a_{y2}] \cdots\cdots (2)$$

Where A_1 and A_2 are the absorbance of sample solutions at 299 and 280 nm, respectively. Cx and Cy are concentrations of LZL and EUG in the sample solution. By substituting the values of A1 and A_2, the Cx and Cy can be calculated by solving equations [19, 20].

Absorbance Ratio Method: In this method, absorbances of both the drugs LZL and EUG were measured at two chosen wavelengths. One of the wavelengths is λ1, the λmax of either drug, and λ2 is the wavelength of the iso-absorptive point of both drugs. At 238nm, both drugs LZL and EUG show absorption as indicated in **Fig. 1A**. Thus, we have selected λ1 as 299 nm and λ2 as 238 nm (iso-absorption point) for substitution in the absorbance equation. After that, we selected λ1 as 280 nm and λ2 as 238 nm (iso-absorption point) for substitution in the equation. The concentration of the individual drugs was determined using the following equations (3) and (4) [21]:

$$Q_m = A_1 / A_2 \times Q_x = ax_2 / ax_1 Q_y = ay_2 / ay_1$$

$$Cx = Qm - Qy \times A_1 / Qx - Qy \times ax_1 \cdots\cdots\cdots\cdots (3)$$

$$Cy = Qx - Qm \times A_1 x - Qx - Qy \times ax_1 \cdots\cdots\cdots\cdots (4)$$

Cx and Cy are concentrations of LZL and EUG respectively ax_1 and ax_2 are absorptivity of LZL at 299 and 238 nm ay_1 and ay_2 are absorptivity of EUG at 280 and 238nm Qm = A_2/A_1, QX = ax_2/ ax1 and QY = ay_2/ay_1

Validation of a Developed Analytical Method of LZL and EUG in Phosphate Buffer pH 5.5: The main objective of validation of the method is to demonstrate that the method is suitable for its intended purpose as per ICH guidelines. The developed method is as per ICH Q2 (R1) guidelines for parameters like linearity, precision (repeatability, intraday, and inter-day precision), accuracy, robustness, the limit of detection, and the limit of quantitation.

Linearity Study: For the linearity study, 1000 µg mL^{-1} solution of LZL and EUG was prepared in PBS pH 5.5 and ethanol (1:1). From the above solution 100 µg mL^{-1} stock solution was prepared using PBS. From LZL and EUG stock solutions 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 aliquots were taken in 10 mL volumetric flasks, and made up to volume with PBS 5.5 to obtain working solutions ranging from 1-20 µg mL^{-1} of each drug.

Precision Study: The precision study of the developed method was evaluated by conducting repeatability, intraday, and inter-day precision studies. For intermediate intra-day precision, 3 different samples of 10 µg mL^{-1} mixture of LZL and EUG solution were analyzed at 3 different time intervals on the same day. Inter-day precision was determined by analyzing 3 different samples of working solutions of 10 µg mL^{-1} mixture of LZL and EUG on three successive days. A repeatability study was conducted by preparing different samples (n = 6) of 10 µg mL^{-1} mixture of LZL and EUG solution. These samples were analyzed and % RSD was calculated

Accuracy Study: The standard addition method was used to study the reliability and validity of the developed method. For accuracy studies, the known concentration of a stock solution (100 µg mL^{-1}) at 3 levels (80%, 100%, and 120%) was spiked into the working solution and percent recovery was calculated. This study was repeated thrice (n=3).

Robustness Study: The robustness of the developed method was studied by analyzing the same working solution of LZL and EUG (10 µg mL^{-1}) by deliberate variation in the method parameters. In this work, we studied the robustness of the method by varying pH and wavelength. Variation in pH was measured by changing the pH from 5.3, 5.5, and 5.7. Variation in wavelength was measured by altering the wavelength for LZL from 297, 299, and 301 nm and for EUG from 278, 280, and 282 nm. % RSD was determined and the study was conducted in triplicate.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD gives the value of the minimum amount of drug that can be reliably detected using the developed analytical method. The LOQ gives the value of the minimum amount of drug which can be reliably quantified using the developed analytical method. The formula for the calculation of LOD is given below:

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where σ is the standard deviation and S is the slope of the regression line.

RESULTS AND DISCUSSION: UV Spectroscopy method development and optimization of chromatographic conditions LZL exhibits maximum absorption at a wavelength (λmax) 280 nm, while EUG exhibits maximum absorption at a wavelength (λmax) 299 nm, and from overlay spectra, it is evident that the iso-absorptive point is 238 nm (Fig. 2). Comparative data of percent recovery of LZL and EUG using linearity method (LM), simultaneous equation method (SEM) and absorption ratio method (ARM) is collated in **Table 1.**

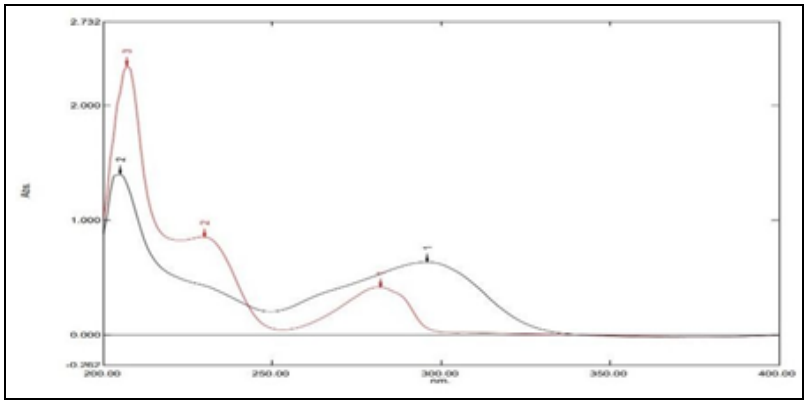

FIG. 2: UV SPECTRUM OF LZL AND EUG USING OPTIMIZED CONDITIONS

TABLE 1: DRUG RECOVERY FOR LZL AND EUG USING LM, SEM, AND ARM

Drugs	Concentration	Drug recovery (µg/mL)			
		LM	SEM	ARM (λ280)	ARM (λ299)
Luliconazole	10ppm	9.4	10.08	10.84	10.44
Eugenol	10ppm	10.5	11.61	11.22	11.64

Validation Studies of the Developed Analytical Method:

Linearity and Calibration Curve: A linearity study was conducted; 6 different concentration solutions of LZL (5-30 µg mL^{-1}) and 8 different concentrations of EUG (5-40 µg mL^{-1}) in methanol. Linearity studies were conducted using 11 different concentrations of LZL and EUG in phosphate buffer pH 5.5. The absorbance was noted and a calibration curve was plotted using absorbance on the Y axis and concentrations on the X axis. The calibration curve of LZL and EUG in methanol is shown in **Fig. 3** and the coefficient of regression was found to be 0.9994 and 0.9992 respectively. The calibration curve of LZL and EUG in phosphate buffer pH 5.5 is shown in **Fig. 4** and the coefficient of regression was found to be 0.999 and 0.9968 respectively. The results of linearity studies of LZL and EUG in methanol and in phosphate buffer pH 5.5 are indicated in **Tables 2** and **3.**

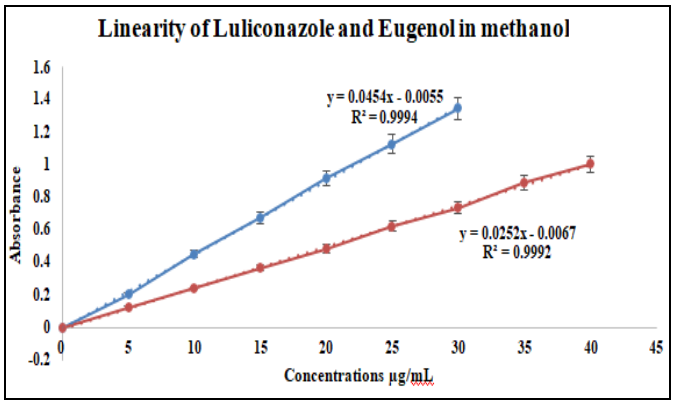

FIG. 3: CALIBRATION CURVE OF LZL AND EUG IN METHANOL

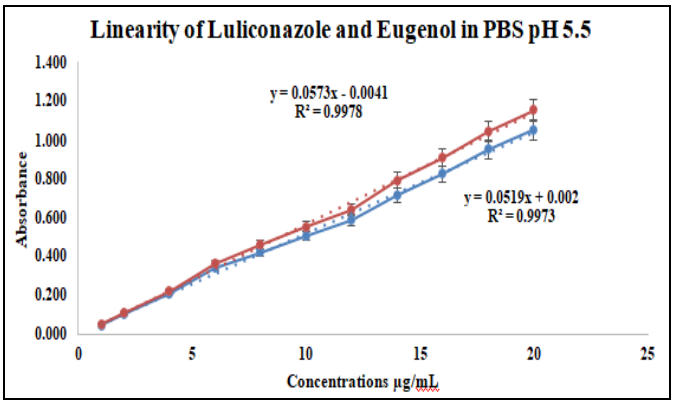

FIG. 4: CALIBRATION CURVE OF LZL AND EUG IN PHOSPHATE BUFFER pH 5.5

TABLE 2: LINEARITY STUDY OF LZL AND EUG IN METHANOL

Parameters	Luliconazole	Eugenol
Linearity range	5-30 µg/mL	5-40 µg/mL
Regression equation	y = 0.0454x - 0.0055	y = 0.0252x - 0.0067
Coefficient of correlation (R^2)	R^2 = 0.9994	R^2 = 0.9992
Slope (m)	0.0454	0.0252
Intercept (c)	-0.0055	-0.0067

TABLE 3: LINEARITY STUDY OF LZL AND EUG IN PHOSPHATE BUFFER pH 5.5

Parameters	Luliconazole	Eugenol
Linearity range	5-30 µg/mL	5-40 µg/mL
Regression equation	y = 0.0443x + 0.0018	y = 0.0223x - 0.0208
Coefficient of correlation(R^2)	R^2 = 0.999	R^2 = 0.9968
Slope (m)	0.0443	0.0223
Intercept (c)	0.0018	-0.0208

Precision Study: The developed method was found to be precise since the results of the precision study showed that the percent relative standard deviation of LZL and EUG was below 2% at all three levels of studies- repeatability, intra- and inter-day precision **Table 4.**

TABLE 4: PRECISION STUDY OF LZL AND EUG

Parameters	Luliconazole		Eugenol	
	Mean Abs.± SD	%RSD	Mean Abs.±SD	%RSD
Repeatability	0.542 ± 0.008	1.440	0.583 ± 0.009	1.610
Inter-day precision	0.559 ± 0.006	1.110	0.595 ± 0.009	1.449
Intra-day precision	0.543 ± 0.009	1.595	0.595 ± 0.007	1.191

*The data is expressed as mean ± SD, n = 6 injections, SD- standard deviation, % RSD- relative standard deviation.

Accuracy Study: An accuracy study was conducted, mean recovery of LZL at EUG was calculated at three different levels (80, 100, and 120%) of addition **Table 5**. The percent recovery was found between 99.42% and 105.16%.

TABLE 5: ACCURACY STUDY OF LZL AND EUG

Drugs	Level	Std amount (µg/mL)	Amountadded (µg/mL)	Total amount (µg/mL)	Amount recovered (µg/mL) ± SD	% Recovery	% RSD
Luliconazole	80%	10	8	18	18.274 ± 0.181	101.52	0.991
	100%	10	10	20	20.599 ± 0.375	102.99	1.822
	120%	10	12	22	22.638 ± 0.091	102.90	0.403
Eugenol	80%	10	8	18	18.930 ± 0.285	105.16	1.504
	100%	10	10	20	20.106 ± 0.227	100.52	1.128
120%	10	12	22	21.874±0.135	99.42	0.615	12%

*The data is expressed as mean ± SD, n = 3 injections, SD- standard deviation, % RSD- relative standard deviation.

Robustness Study: The robustness of the developed method was evaluated by making small variations in the wavelength and pH **Table 6.**

TABLE 6: ROBUSTNESS STUDY OF LZL AND EUG

Variable	Level	Luliconazole		Level	Eugenol	
		Mean Abs. ±SD	% RSD		Mean Abs. ± SD	% RSD
Variation in wavelength	297	0.529 ± 0.009	1.637	278	0.541 ± 0.009	1.573
	299	0.534 ± 0.01	1.798	280	0.573 ± 0.006	0.992
	301	0.550 ± 0.008	1.549	282	0.592 ± 0.012	1.978
Variation inpH	5.3	0.550 ± 0.010	1.734	5.3	0.562 ± 0.009	1.533
	5.5	0.548 ± 0.006	1.174	5.5	0.562 ± 0.009	1.596
	5.7	0.558 ± 0.01	1.769	5.7	0.565 ± 0.006	1.036

*The data is expressed as mean ± SD, n=3 injections at each level, SD- standard deviation, %RSD- relative standard deviation.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD and LODQ were calculated using the slope of the regression coefficient equation. The LOD values of LZL and EUG were found to be 0.826 and 0.863 µg/mL respectively. The LOQ value of LZL and EUG was found to be 2.504 and 2.617 µg/mL respectively [22].

CONCLUSION: A simple, easy-to-use UV spectrophotometric method was successfully developed for the simultaneous analysis of Luliconazole and Eugenol. We have developed simultaneous and absorption ratio methods for the quantitative estimation of LZL and EUG. There is no significant difference found in the recovery of drugs using both methods. The methods were validated on the parameters as per ICH guidelines. The results obtained were reproducible with no interference and the developed method was found to be linear, precise, accurate, and robust and can be used for quantitative determination of Luliconazole and Eugenol in combination formulations.

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CONFLICT OF INTEREST: The authors declare no conflict of interest

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