IJPSR (2020), Volume 11, Issue 9 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 04 October 2019; received in revised form, 17 February 2020; accepted, 11 March 2020; published 01 September 2020

DEVELOPMENT AND VALIDATION OF A CHROMATOGRAPHIC ASSAY METHOD FOR THE DETERMINATION OF LULICONAZOLE IN CREAMS

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Keywords:

Luliconazole, Binary gradient HPLC, Validation, ICH Guidelines Stability-indicating

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ABSTRACT: A simple and rapid reverse-phase high-performance liquid chromatographic technique was developed and validated for the determination of Luliconazole in the presence of its excipients. Chromatographic elution was performed on a binary gradient HPLC equipped with PDA detector using a Luna-5µ C8(2) 100 Å column (250 × 4.6 mm, 5 µm) with orthophosphoric acid (0.1%) and methanol (20:80% w/v) as mobile phase at a flow rate of 1.2 mL/min. The method follows Beer-Lambert's law over a concentration range 0.1-200 µg/mL (y = 53981x + 25076, R^2 = 0.999). The LOD and LOQ were found to be 0.068 µg/mL and 0.206 µg/mL indicating the sensitivity of the method with the required precision and accuracy. The method proved to be specific as there was no interference from the commonly used excipients in a formulation like methylparaben and also in the presence of degradants when exposed to a variety of stress conditions (acidic, alkaline, oxidation, thermal, hydrolytic and photolytic degradations). It was observed that Luliconazole was more sensitive towards alkaline conditions, and hence the proposed method, validated as per ICH guidelines proved to be stability-indicating and can be successfully applied for the determination of Luliconazole in creams.

INTRODUCTION: Luliconazole (LCZ), an anti-fungal agent [1] is chemically named as (2E)-2-[(4R)- 4- (2, 4-dichlorophenyl)-1, 3-dithiolan-2-ylidene]- 2- imidazol- 1- yl- acetonitrile. It has a molecular formula $C_{14}H_9C_{l2}N_3S_2$ with a molecular weight of 354.28 g/mol. and melting point in the range 121-125 °C. Luliconazole is soluble in organic solvents such as ethanol, DMSO, dimethyl formamide (DMF).

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.11(9).4622-28

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(9).4622-28

It is a broad-spectrum imidazole that is active against various fungi, including *Tinea candida*, *Aspergillus*, *trichophyton* and *Epidermophyton* is used for the treatment of interdigital tinea pedis, tinea cruris, and tinea corporis. Although the exact mechanism of action against dermatophytes is unknown, Luliconazole appears to inhibit ergosterol synthesis by inhibiting the enzyme lanosterol demethylase. Inhibition of this enzyme's activity by azoles results in decreased amounts of ergosterol, a constituent of fungal cell membranes, and a corresponding accumulation of lanosterol [2, 3]. Luliconazole as a 1% topical cream is indicated for the treatment of athlete's foot, jock itch, and ringworm caused by dermatophytes such as *Trichophyton rubrum*, *Microsporum gypseum* and *Epidermophyton floccosum* [4, 5].

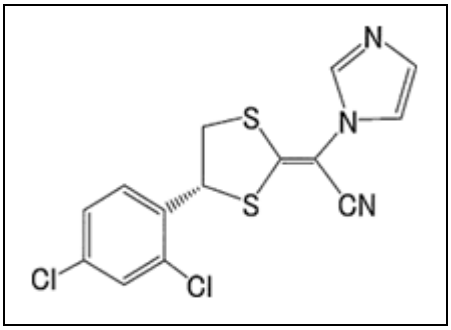


FIG. 1: STRUCTURE OF LULICONAZOLE

Review of literature for Luliconazole analysis revealed existing methods which include an LC-MS/MS method [6], a HPTLC method [7], a few HPLC methods [8, 9,] and few spectroscopic [10, 11] methods for the quantification of Luliconazole and its related substances in formulations and biological samples. Since very few HPLC methods were reported, an attempt has been made to develop and validate a simple and rapid RP-HPLC method for the assay of Luliconazole in creams.

MATERIALS AND METHODS:

Chemicals and Reagents: Reference standard of Luliconazole was obtained as a gift sample from Sun Pharma Ltd. and the pharmaceutical dosage form (Lulifin® cream) containing 1% w/w of Luliconazole was procured from a local pharmacy. Methanol (HPLC grade), hydrochloric acid, sodium hydroxide, hydrogen peroxide, orthophosphoric acid and methylparaben were procured from Merck Life Science Private Limited. HPLC grade water was used throughout the study.

HPLC Instrumentation and Conditions: Shimadzu Model CBM-20A/20 Alite HPLC system equipped with SPD M20A prominence photodiode array detector and Luna-Su C8(2) 100 Å column (250× 4.60 mm, 5 µm particle size) was used for chromatographic separation. Isocratic mode of elution was selected using orthophosphoric acid (0.1%) and methanol (20:80, v/v) mixture as the mobile phase at a flow rate of 1.2 mL/min. The UV detection was carried out at 294 nm and the overall run time was 10 min.

Preparation of Stock Solution and Working Standard: Stock solution (1000 µg/mL) was prepared by dissolving 25 mg of Luliconazole in methanol in a 25 mL volumetric flask. Working standard solutions were prepared from the stock solution with the mobile phase as diluent.

Preparation of Ortho Phosphoric Acid (0.1%): 1mL of orthophosphoric acid was taken and dissolved in 1000 mL HPLC water in a 1000 ml volumetric flask.

Preparation of Sample Solution (Luliconazole in Cream): For the analysis of Luliconazole in creams, 0.5gm of cream (1% w/w) was taken in a 50 mL volumetric flask, methanol was added in increments, the drug was dissolved by constant stirring and after complete dissolving of the cream base, remaining volume was made up with methanol and filtered.

Validation: The developed method was validated as per ICH (2005) validation[12] parameters such as linearity, precision, accuracy, the limit of quantitation (LOQ), the limit of detection (LOD), specificity, and robustness.

Linearity: Linearity of the assay method was conducted by preparing a series of solutions (0.1–200 µg/mL) from the standard working solution, and each solution was injected into the HPLC system in triplicates. The average peak area of the chromatograms obtained was plotted against concentration to construct the calibration curve.

Accuracy: The accuracy of the assay method was performed by standard addition at three concentration levels (50, 100, and 150%). Standard solution (5, 10, 15 µg/mL) of Luliconazole was added to a pre-analyzed sample solution of cream, each solution was evaluated in triplicate, and the percentage recovery was calculated.

Precision: The precision study was conducted in terms of repeatability (intra-day) and intermediate precision (inter-day). The intra-day precision studies were carried out at three different concentrations (30, 50, and 70 µg/mL), and the % RSD was calculated. The inter-day precision study was also performed for the same concentrations on two different days *i.e.,* day 1, day 2, and the % RSD was calculated.

Robustness: Robustness study was performed by incorporating small changes in the method parameters such as wavelength (292 and 296 nm), the composition of mobile phase (75 and 85% of organic phase) and flow rate (1.1 and 1.3 mL/min). A standard and sample solution of Luliconazole (20

µg/mL) was injected in replicates under the altered chromatographic conditions, and the peak area along with system suitability parameters was monitored.

Limit of Quantification and Limit of Detection: The limit of quantification and limit of detection were based on the standard deviation of the response and the slope of the constructed calibration curve (n=3), as described in ICH guidelines Q2 (R1) (ICH guidelines, 2005). The sensitivity of the method was established with respect to the limit of detection LOD and LOQ for analytes.

Specificity: The specificity of the method was established in the presence of excipients and degradation products. The interference of the commonly used additives in creams was determined by injecting a solution of methyl-paraben (10 µg/mL) along with the drug into the column. The blank, standard, sample, and specificity chromatograms obtained were analyzed.

Forced Degradation Studies: The stability [12, 13,] indicating properties and specificity of the method were evaluated by performing forced degradation studies (ICH, 2003). The stress studies were conducted at an initial concentration of 100 µg/mL of Luliconazole.

Acidic degradation was conducted by exposing a 100 µg/mL. Luliconazole solution to 1ml of 1 N hydrochloric acid for 30 min at 60 ºC. The stressed sample was cooled, neutralized, and diluted with the mobile phase. Similarly, alkaline stress degradation study was conducted by adding 0.1mLof 0.1 N NaOH to the drug solution and left at room temperature for one hour. This solution was neutralized prior to dilution with the mobile phase. Hydrolytic stress studies were conducted using 1mL of HPLC water, and the drug solution was heated at 60 ºC, cooled, and diluted with the mobile phase. Oxidative stress studies were conducted using 1 mL of 30 % H_2O_2 on the drug solution, heated to 60 ºC for 30 min. Thermal stress studies on Luliconazole were conducted in a thermostat maintained at 70 ºC for 30 min. Photolytic degradation study was performed by exposing the drug solution (100 µg/mL) to UV light at 365 nm in a UV chamber for 3 h and later diluted with the mobile phase. All the above solutions were filtered prior to an injection using syringe filters, and 20 µL of each solution was injected in triplicates in to the HPLC system. The obtained chromatograms were analyzed in the presence of degradants. System suitability parameters were checked for each degradation study, and the assay values were calculated.

Assay: The developed method was also applied for the determination of Luliconazole in marketed formulations. Lulifin® (cream) containing 1%w/w of Luliconazole was selected, and the sample solution was prepared as discussed above. From the filtrate, suitable aliquots were diluted with the mobile phase and analyzed using the calibration curve.

RESULTS AND DISCUSSION: A simple and rapid HPLC method has been developed and validated for the assay of Luliconazole in creams. Initially, various mobile phases were tried to achieve better resolution and separation conditions. The drug solution was analyzed using different conditions such as water: methanol (30:70 v/v, 1 mL/min.), water: methanol (20:80 v/v, 1.2 mL/min.) in which the peak shapes were not symmetrical and also did not satisfy the system suitability parameters. Then the mobile phase composition was changed to orthophosphoric acid (0.1 %): methanol (20: 80, v/v) where a peak was eluted at 3.59 ± 0.02 min. which was sharp without tailing (UV detection at 294 nm) and so these parameters were taken as the optimized chromatographic conditions. The representative chromatogram of the standard drug solution is shown in **Fig. 2**. The developed method was validated for system suitability, linearity, the limit of quantitation (LOQ), the limit of detection (LOD), precision, accuracy, selectivity, and robustness as per the ICH guidelines.

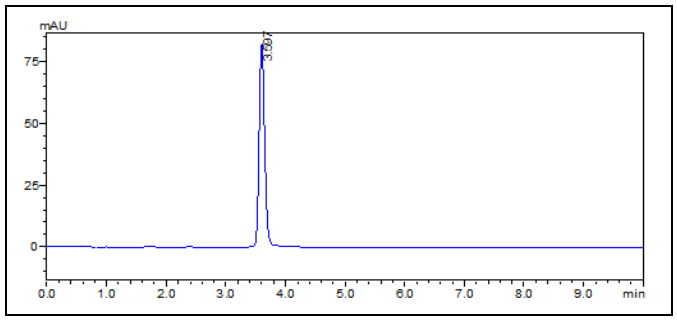


FIG. 2: CHROMATOGRAM OF LULICONAZOLE (STANDARD)

System Suitability: The system suitability test was performed to ensure that the complete testing system was suitable for the intended application. The parameters measured were peak area, retention time, tailing factor, capacity factor, and theoretical plates. In all measurements, the peak area varied less than 2.0%, and the average retention time was at 4.74 ± 0.21 min. The capacity factor was more than 2, theoretical plates were 6163 ± 150 (more than 2000), and the tailing factor was less than 1.2 for the Luliconazole peak.

Linearity: Luliconazole obeyed linearity over a concentration range of 0.1-200 µg/mL with a linear regression equation of y = 53981x + 25076 (R^2 = 0.999). The linearity data and calibration curve are given in **Table 1** and **Fig. 3**. The LOQ was found to be 0.206 µg/mL, and LOD was found to be 0.068 µg/mL.

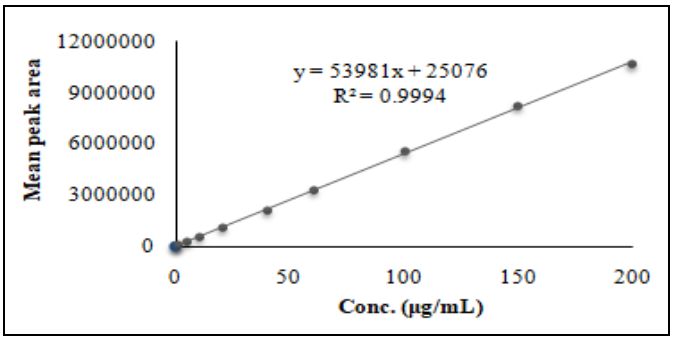


FIG. 3: CALIBRATION CURVE

TABLE 1: LINEARITY DATA

Conc. (µg/mL)	*Peak area ± SD	%RSD	Conc. (µg/mL)	*Peak area ± SD	%RSD
0.1	6163 ± 41.14	0.21	40	2111739 ± 10932.21	0.44
1	53771 ± 88.29	0.33	60	3336344 ± 20119.11	0.21
5	277122 ± 118.79	0.13	100	5551995 ± 24932.92	0.26
10	538520 ± 285.21	0.34	150	8260390 ± 27743.48	0.11
20	1134258 ± 1073.53	0.26	200	10645181 ± 31659.87	0.34

*Mean of three replicates

Accuracy: The method was found to be accurate, as observed from the percentage recovery in the range of 98.1-100.3, as discussed in **Table 2**. The % RSD was found to be 0.101-0.905.

TABLE 2: RECOVERY DATA

Level (%)	Std. Drug (µg/mL)	Formulation (µg/mL)	*Peak area	Conc. (µg/mL)	*Recovery ± SD (%RSD)
50	5	10	824534	14.81	98.2 ± 743.36 (0.5)
100	10	10	1099297	19.91	99.91 ± 53.94 (0.1)
150	15	10	1366503	24.9	99.4 ± 6049.74 (0.9)

*Mean of three replicates

TABLE 3: INTRA AND INTERDAY PRECISION STUDY

Conc. (µg/mL)	Intraday precision *Peak area ± SD (%RSD)	Inter day precision *Peak area ± SD (%RSD)
30	1539064 ± 29865.3 (0.1)	1527098 ± 25716.2 (0.2)
50	2734717 ± 62707.1 (0.4)	2878832 ± 65561.1 (0.1)
70	4002635 ± 46040.9 (0.09)	3852355 ± 59723.5 (0.1)

*Mean of three replicates

Precision: The repeatability of the method was studied in terms of intraday and interday precision in which the % RSD values calculated for the peak areas were obtained as 0.1-0.21 and 0.12-0.21, respectively (<2%). **Table 3** indicates the results representing that the method is precise.

Robustness: Slight changes in flow rate, mobile phase composition, and wavelength were made, and they did not much affect the system suitability parameters like retention time, tailing factor, and theoretical plates, as given in **Table 4**.

TABLE 4: ROBUSTNESS STUDY

Parameter	Condition	Sample peak area	Standard peak area	Tailing factor	Theoretical plates	*Assay ± SD (%RSD)
Flow rate (± 0.1 mL)	1.1 1.2 1.3	1258315	1134258	1.102	6219	100.16 ± 0.7 (0.7)
Mobile phase composition (± 5 parts)	75:25 80:20 85:15	1121329		1.182	6220	100.21 ± 0.96 (0.96)
Wavelength (± 2 nm)	292 294 296	1118743		1.204	6253	99.6 ± 0.63 (0.62)

*Mean of three replicates

Specificity: The method was tested for its specificity in the presence of methylparaben as an additive and it did not pose any interference as observed in the chromatogram given in **Fig. 4**. The methylparaben peak (3.004 min) was well resolved from the drug peak (3.542 min), indicating the specificity of the method.

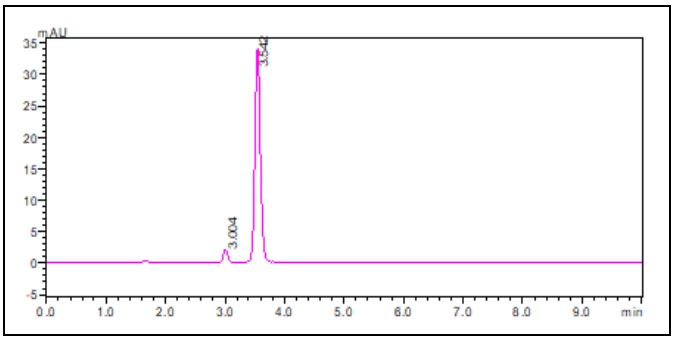


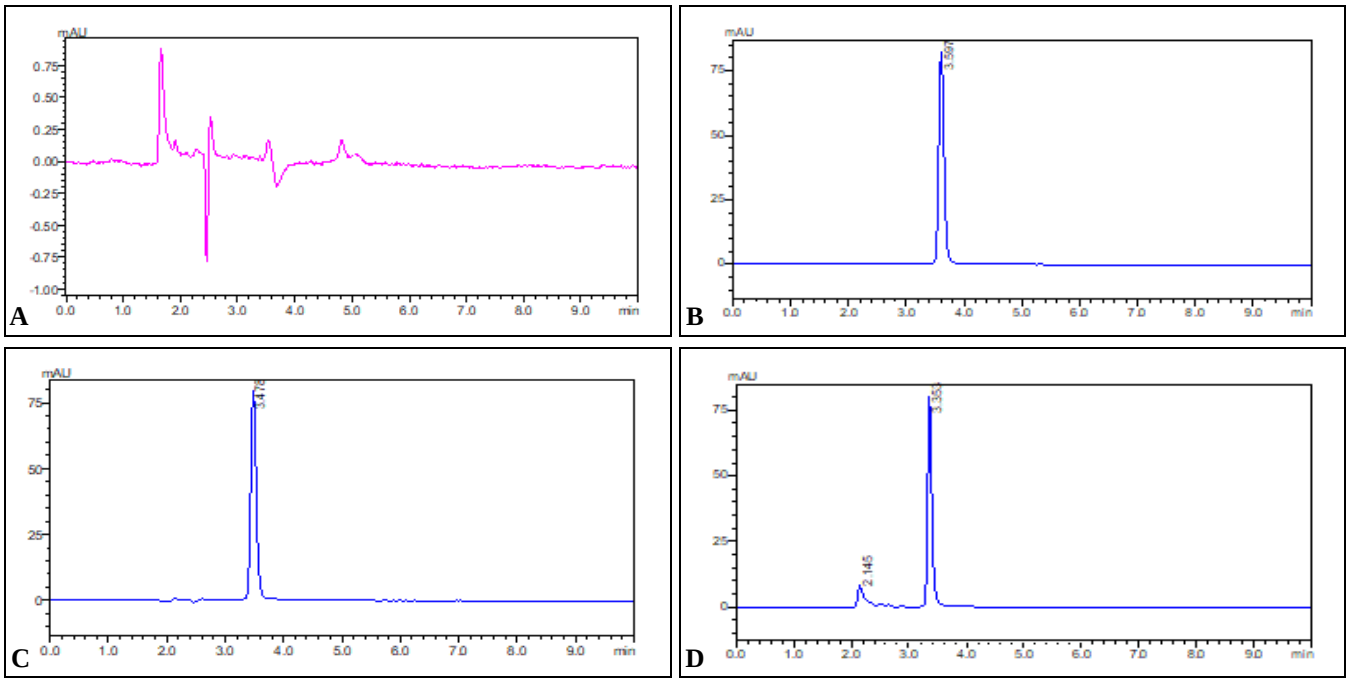
FIG. 4: CHROMATOGRAM OF METHYLPARABEN AND LULICONAZOLE

Forced Degradation Studies: Luliconazole standard drug solutions were exposed to different stress conditions and the drug showed mild degradation in acidic (1.69%) and moderate degradations in oxidation (4.84%), thermal (9.69%), hydrolytic (7.85%) and photolytic (7.85%) stress conditions. The drug was found to be sensitive to alkaline stress conditions, as observed from the degradation behavior (22.27%) given in **Table 5**. The imidazole moiety present in the Luliconazole chemical structure may be responsible for the alkaline degradation for which the extra peak, which was completely resolved from the analyte peak might have eluted at 2.145 min. The typical blank and analyte chromatograms obtained during the assay of stressed samples are shown in **Fig. 5A-F**. The system suitability parameters for the Luliconazole peak in all the stressed conditions showed that the theoretical plates were more than 2000, and the tailing factor was less than 2.0 depicting the stability-indicating nature of the RP-HPLC method. The method also proved to be specific to Luliconazole in the presence of degradants.

TABLE 5: FORCED DEGRADATION STUDY

Stress condition	*Peak area	Theoretical plates	Tailing factor	Resolution	Drug recovered (%)	Drug decomposed (%)
Standard	565967	5989.20	1.223	-	99.99	-
Acidic	556665	5462.11	1.266	-	98.3	1.69
Alkaline	439913	7632.76	1.462	6.74	77.72	22.27
Oxidative	538520	7230.15	1.260	-	95.15	4.84
Thermal	511555	6864.23	1.249	-	90.3	9.69
Photolytic	516063	6921.71	1.273	-	91.1	8.89
Hydrolytic	521520	6684.27	1.223	-	92.14	7.85

*Mean of three replicates



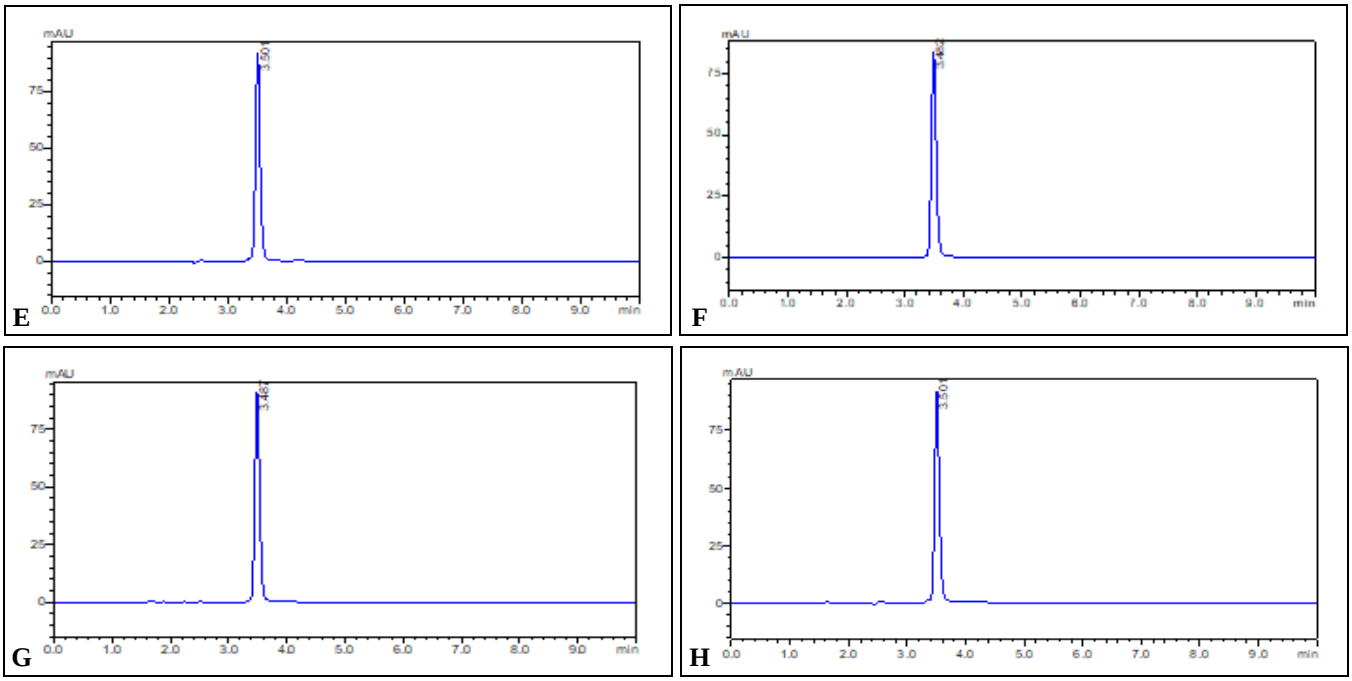


FIG. 5: FORCED DEGRADATION SPECTRUM: TYPICAL CHROMATOGRAMS OF LULICONAZOLE BLANK (A), STANDARD (B), ACID (C), ALKALINE (D), OXIDATIVE (E), THERMAL (F), PHOTOLYTIC (G) AND HYDROLYTIC (H) STRESS

The method was applied for the quantification of Luliconazole in marketed creams, and the assay was obtained as 99.9 ± 1.17% w/w as stated against the label claim given in **Table 6**.

TABLE 6: ASSAY OF LULICONAZOLE IN CREAMS

Brand	Label claim (% w/w)	Amount obtained* (% w/w)	Assay (% w/w) ± SD
LULIFIN	1.0	0.99	99.9 ± 1.17

CONCLUSION: A simple, rapid, sensitive, and reliable RP-HPLC method with good precision and accuracy was developed and validated for the analysis of Luliconazole. The proposed method was specific while estimating the commercial formulations without the interference of excipients and other additives. Also, the method was proved to be stability-indicating and hence can be used for the routine determination of Luliconazole in API and creams.

ACKNOWLEDGEMENT: The authors are grateful to M/s GITAM University, Visakhapatnam (India), for providing necessary research facilities.

CONFLICTS OF INTEREST: The authors declare nil conflict of interest.

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How to cite this article:
Gummadi S and Kommoju M: Development and validation of a chromatographic assay method for the determination of luliconazole in creams. Int J Pharm Sci & Res 2020; 11(9): 1000-07. doi: 10.13040/IJPSR.0975-8232.11(9).4622-28.