Estimation of Luliconazole in Formulation and Biofluid

Abstract

A new, simple, rapid and economic High Performance Thin Layer Chromatography (HPTLC) method was developed and validated for quantitative determination of Luliconazole in pharmaceutical dosage forms. The Rf value of the drug was 0.62 ± 0.05 using Toluene: Methanol: Ethyl Acetate (6:2.5:0.5 v/v/v) as the mobile phase at 300nm. Linearity was obtained within a concentration range of 100-600ng/band with regression coefficient 0.983. The accuracy of the proposed method for analyzing the API in terms of percentage recovery was 99.91 with percentage RSD 0.47%, both of which were within the specified limits. LOD and LOQ were 27ng/band and 83ng/band, respectively. The recovery after application to biological fluid was 100.36 with percentage RSD 0.69% proving its applicability to biological analysis. Mass spectrometric characterization of the samples resulted in a spectrum showing molecular ion peak at 355.1m/z, resulted in direct confirmation in identifying the drug. The experimental data was statistically analysed by one way ANOVA (F-test) and student’s t test which proves that, the developed chromatographic method is precise and accurate and can be used for routine analysis of Luliconazole. This HPTLC method seems to be convenient as well as less time consuming and thus illustrating its wide applicability for the estimation of Luliconazole in bulk drug, cream formulation and biological fluid.

Keywords: HPTLC; Luliconazole; Biological fluid

Abbreviations: HPTLC: High Performance Thin Layer Chromatography; LLCZ: Luliconazole; ANOVA: Analysis of Variance

Introduction

Luliconazole is a novel topical antifungal imidazole with broad-spectrum and potent antifungal activity used in treatment of superficial mycoses. Superficial mycoses are not fatal, but they constitute a serious problem for patients' quality of life in view of the considerable discomfort and/or cosmetic deformity they cause. These diseases are found worldwide and affect 20 to 25% of the world's population [1]. Dermatophytosis is the most common infection among the superficial mycoses [2-8]. Luliconazole is a novel antifungal drug launched in India by Ranbaxy Laboratories Ltd. The compound was originally screened from active compounds related to lanoconazole, a potent antidermatophytic drug. Currently, a 1% cream and a 1% formulation, Lulifin (Ranbaxy Laboratories Ltd., Gurgaon, Haryana, India) were in the market. A reference standard, Luliconazole chemically, 4-(2,4-dichlorophenyl)-1,3-dithiolan-2-ylidene-1-imidazolylacetonitrile (Figure 1), was kindly supplied by Ranbaxy Laboratories Ltd. (Gurgaon, Haryana, India). The cream formulation, Lulifin™ (Ranbaxy Laboratories Ltd., Gurgaon, Haryana, India) composed of 10 mg of Luliconazole in each gram, was purchased in the market. Silica Gel 60 F254 TLC plates (10 X 10 cm, layer thickness 0.2 mm, E. Merck, Darmstadt, Germany) were used as stationary phase. Methanol, Ethyl acetate and Toluene (AR grade, Fisher Scientific, India) were used for mobile phase preparation.

Materials and Methods

Chemicals, reagents and Materials

A reference standard, Luliconazole chemically, 4-(2,4-dichlorophenyl)-1,3-dithiolan-2-ylidene-1-imidazolylacetonitrile (Figure 1), was kindly supplied by Ranbaxy Laboratories Ltd. (Gurgaon, Haryana, India). The cream formulation, Lulifin™ (Ranbaxy Laboratories Ltd., Gurgaon, Haryana, India) composed of 10 mg of Luliconazole in each gram, was purchased in the market. Silica Gel 60 F254 TLC plates (10 X 10 cm, layer thickness 0.2 mm, E. Merck, Darmstadt, Germany) were used as stationary phase. Methanol, Ethyl acetate and Toluene (AR grade, Fisher Scientific, India) were used for mobile phase preparation.

HPTLC method and chromatographic conditions

Sample application: The standard and formulation samples of Luliconazole were spotted on Precoated TLC plates in the form of narrow bands of lengths 6mm, with 8.0mm from the bottom and left margin 15 mm and with 10mm distance between two bands. Samples were applied under continuous drying stream of nitrogen gas at constant application rate.
Mobile phase and migration

Plates were developed using mobile phase consisting of Toluene: Methanol: Ethyl Acetate (6:2.5:0.5 v/v/v). Linear ascending development was carried out in 20cm × 20cm twin trough glass chamber equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 20min at 25±2°C. Ten milliliters of the mobile phase (5mL in trough containing the plate and 5mL in other trough) was used for each development and allowed to migrate a distance of 70mm, which required 10min. After development, the TLC plates were dried completely.

Densitometric analysis and quantitation procedure

Densitometric scanning was performed on Camag TLC scanner III in absorbance mode and operated by winCATS planar chromatography version 1.4.2.8121. The source of radiation utilized was deuterium lamp. The spots were analyzed at a wavelength of 300nm. Concentrations of compound chromatographed was evaluated as peak areas against concentrations using linear regression equation.

Preparation of luliconazole standard stock solution

Stock solution was prepared by weighing Luliconazole (10mg). Weighed powder was accurately transferred to a volumetric flask of 10mL and dissolved in and diluted to the mark with methanol to obtain a standard stock solution of Luliconazole (1mg/mL).

Method validation: Validation of the developed HPTLC method in bulk, cream was carried out as per the ICH Q2 (R1) guideline [17] and method was also applied to biological fluid. The experimental data was statistically analysed by one way ANOVA for intraday and interday precision and F values were calculated at each level, medium and high conc. level. Student’s t test was applied to accuracy data and t value was calculated.

Specificity: The specificity of the developed method was established analyzing the sample solutions containing Luliconazole from marketed formulations in relation to interferences from formulation ingredients and biological fluid. The spot for Luliconazole in the sample was confirmed by comparing Rf values of the spot with that of the standard.

Sensitivity: Sensitivity of the method was determined with respect to limit of detection (LOD) and limit of quantitation (LOQ) by using the Standard Deviation of the Response and the Slope. The slope (S) was estimated from the calibration curve of the analyte and the standard deviation of y-intercepts of regression lines was used as the standard deviation (σ) based on Calibration Curve method. Six sets of Series of concentrations of drug solutions (100–600ng/spot) were applied on plate and analyzed to determine LOD and LOQ. LOD was calculated as 3 times the noise level, and LOQ was calculated as 10 times the noise level.

Linearity and calibration curve: Linearity of the method was evaluated by constructing calibration curves at six concentration levels. Calibration curves were plotted over a concentration range of 100–600ng/spot. Aliquots of standard working solution of Luliconazole were applied to the plate (1, 2, 3, 4, 5, and 6µL/spot). The calibration curves were developed by plotting peak area versus concentrations (n = 6) with the help of the winCATS software.

Accuracy: Accuracy of the method was evaluated by carrying the recovery study at three levels. Recovery experiments were performed by adding three different amounts of standard drug, that is, 80%, 100%, and 120% of the drug, to the preanalyzed formulation solution. Data was analyzed and t value was calculated by student’s t test.

Precision: Precision was evaluated in terms of Intraday and Interday precisions. Intraday precision was determined by analyzing sample solutions of Luliconazole from at three levels covering low, medium, and higher concentrations of calibration curve for three times on the same day. Interday precision was determined by analyzing sample solutions of Luliconazole at three levels covering low, medium, and higher concentrations. Mean, relative standard deviation (RSD) and F values of peak area were obtained.

Robustness: By introducing small changes in mobile phase composition, chamber saturation time, Change in slit width, Change in Wavelength, Change in scan speed Robustness of the method was determined in (n=3) at a concentration level of 300ng/spot and %RSD of peak area was calculated.

Analysis of cream formulation

An appropriate portion of 0.1g of Lulifin™ was weighed and transferred into a 10mL volumetric flask using weight by transfer method. Further, 5 mL of methanol was added and was shaken, till the cream base gets dissolved. Finally the volume was adjusted to the mark with methanol to get final concentration of 1mg/ml. As such three samples were prepared. The mixture were filtered immediately with Whatman filter paper no. 41 and subjected to chromatographic analysis. The drug peak area was referred to the regression equation to get the sample concentration and % nominal label claimed.

Application of the proposed method for estimation of luliconazole in biological fluid

0.25ml of biological fluid was deproteinated by acetonitrile and spiked with working solutions to produce desired concentrations of luliconazole. The precipitated mixture was shaken for 20sec on a Vortex Shaker at maximum speed followed by centrifugation at 10,000rpm for 1 min at room temperature. By using the same procedure the sample was prepared and analyzed in triplicate. μL

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volume of sample were applied on HPTLC plate to produce 300ng/ml of sample under above conditions. The spiked specimens were then processed using the aforementioned procedures and analysed. Specificity study showed that the components of biological matrix did not interfere with the analyte, thereby confirming the specificity of the proposed extraction procedure (Figure 2). The recovery of Luliconazole was calculated by comparison of the peak area of 300ng/ml prepared in biological fluid (extracted) with unextracted Luliconazole.

**Characterization by mass spectrometry:** The triple quadrupole system was an API 4000 Q TRAP (ABSCIEX, CA, USA) LC-MS/MS spectrometer fitted with an electro spray ionization interface. The ESI-MS was operated in both positive and negative detection mode. Calibration of the mass analyzer was performed by infusion (10µl min⁻¹) of a commercial mixture of polypropylene glycol (PPG) which was supplied by AB SCIEX using a 1ml Hamilton syringe and monitored eight mass-to-charge ratios (m/z) in the 59-1800 mass range. The ESI source conditions were: ion spray voltage, 5500 V; nebulizer gas (GS1), 50 psi; curtain gas, 25 psi; turbo gas (GS2), 50 psi; collision gas (CAD), 7 psi and ion source temperature 470°C.

**Results**

**HPTLC method optimization and validation**

To optimize the HPTLC parameters, several mobile phase compositions were tried. Satisfactory development of Luliconazole was obtained with mobile phase consisting of toluene- methanol-ethyl acetate (6.0:2.5:0.5 v/v/v) as mobile phase, It was observed that Luliconazole showed maximum absorption at 300 nm (Figure 3), hence the densitometric scanning was performed at 300.00nm for all the measurements. The mobile phase enabled good resolution, a sharp and symmetrical peak of $R_f=0.62±0.05$ (Figure 4) from a compact and non diffuse band. Linearity and range: The linear regression data revealed a good linear relationship over the concentration range of 100-600ng/band with correlation coefficient ($r^2=0.983$), shown in (Figure 5). The results are showed in Table 1.

**Table 1:** Calibration Parameters of Luliconazole for HPTLC.

| Parameter                  | Luliconazole |
|----------------------------|--------------|
| Linearity range            | 100-600ng/band |
| Slope (m)                  | 15.02        |
| Intercept (c)              | 2119         |
| LOD (ng/band)              | 27ng/band    |
| LOQ (ng/band)              | 83ng/band    |
| Intra-day (Mean of n=9) %RSD | 0.94%       |
| Inter-day (Mean of n=18) %RSD | 2.16%      |

**Abbreviations:** LOD: Limit of Detection; LOQ: Limit of Quantitation; RSD: Relative Standard Deviation

**Precision:** The proposed method was found to be precise as indicated by % RSD, when the results of intra-day and inter-day were subjected to one-way ANOVA and F values were calculated at each QC level, the F values were found to be less than the tabulated F values. This indicated that there was no significant difference
between intra- and inter-day variability at p<0.01 as shown in Table 2 & 3, suggesting good intermediate precision.

Table 2: Results of method precision Intraday for Luliconazole (n=6).

| Conc. (ng/Band) | 100(ng/Band) | 300 (ng/Band) | 600(ng/Band) |
|----------------|--------------|---------------|--------------|
| Intraday 1     | 3222         | 6782          | 10781        |
| Intraday 2     | 3187         | 6877          | 10567        |
| Intraday 3     | 3208         | 6948          | 10598        |
| Mean±S.D.      | 3206±17.59   | 6869±83.70    | 10649±115.65 |
| ANOVA(F-value) | 0.73         | 1.20          | 5.72         |
| %RSD           | 0.54         | 1.20          | 1.08         |
| Mean %RSD     | 0.94         |               |              |

The value for $F_{(calculated)}$ is lower than the $F_{(tabulated)}$, indicating no significant difference between the intraday variability at p<0.01.

Abbreviations: SD: Standard Deviation; RSD: Relative Standard Deviation; ANOVA: Analysis of Variance

Table 3: Results of method precision Inter-day for Luliconazole (n=6).

| Conc.(ng/Band) | 100 (ng/Band) | 300 (ng/Band) | 600 (ng/Band) |
|----------------|--------------|---------------|--------------|
| Interday 1     | 3008         | 6330          | 10010        |
| Interday 2     | 3060         | 6632          | 10261        |
| Interday 3     | 3041         | 6540          | 10087        |
| Mean±S.D.      | 3036±43.50   | 6501±203.56   | 10119±195.08 |
| ANOVA (F-Value)| 2.65         | 6.18          | 3.30         |
| %RSD           | 1.43         | 3.13          | 1.92         |
| Mean %RSD     | 2.16         |               |              |

The value for $F_{(calculated)}$ is lower than the $F_{(tabulated)}$, indicating no significant difference between the interday variability at p<0.01.

Abbreviations: SD-Standard Deviation, RSD-Relative Standard Deviation, ANOVA-Analysis of Variance

Accuracy: The proposed method when used for the estimation of Luliconazole from pharmaceutical dosage form after spiking with the standard, afforded recovery of 99.56-100.51% at different levels was found. The results obtained by the proposed method were statistically compared with those of the literature method. [15] By applying the Student’s t-test for accuracy, the calculated t-values ($t=1.68$) was found to be less than the tabulated value, $t= 4.303$ at 95% confidence level for two degrees of freedom suggesting that the proposed method and the literature method [15] do not differ significantly with respect to accuracy. The results of analysis are depicted in Table 4.

Limit of detection (LOD) and limit of quantification (LOQ): The limit of detection and limit of quantification was 27ng/ band and 83ng /band respectively.

Robustness: Robustness was checked by making a slight deliberate change in the experimental parameters i.e. change in Mobile phase composition, Change in saturation time, change in scan speed, change in wavelength and chromatogram was run. Robustness of the method was checked at middle concentration of 300ng/spot. The method was found to be robust since the peak area values were not significantly affected. The results of analysis are depicted in Table 5.

Specificity: The method was found to be specific since no interferences spots were seen when carried out in presence of additives. The spot for Luliconazole in the sample was confirmed by comparing R$_f$ values of the spot with that of the standard. As shown in Figure 6 & 7. As active constituent is soluble in methanol where as additives are in soluble in methanol.

Recovery in biological fluid: The recovery of Luliconazole in biological fluid for HPTLC at the concentration 300ng/spot in triplicates were found to be 100.36% as shown in Table 6. There is no interference of the biological fluid in the quantitation of Luliconazole as shown in Figure 8. There were no changes in R$_f$ of Luliconazole, hence the method is selective.

Mass spectrometry: Mass spectrometric analysis of the samples resulted in a spectrum showing molecular ion peak at 355.1 m/z. Mass spectrometric characterization resulted in direct confirmation in identifying the drug as shown in (Figure 2)
Table 4: Results of accuracy of Luliconazole and Statistical comparison with Literature method.

| Recovery Level | Initial Amount (ng/Band) | Conc. of Standard Drug added (ng/Band) | Mean* % Recovery ±S.D. | %RSD | t-Value |
|----------------|--------------------------|---------------------------------------|------------------------|-------|---------|
| 80%            | 200                      | 160                                   | 99.56 ± 0.317          | 0.32  | 1.68    |
| 100%           | 200                      | 200                                   | 100.51 ± 1.010         | 1     |         |
| 120%           | 200                      | 240                                   | 99.67 ± 0.0945         | 0.09  |         |
| Mean Recovery  |                          |                                       | 99.91±0.473            | 0.47  |         |

(no. of determinations=3 per conc.) for HPTLC.*Average of three determinants. There was no significance difference between the methods using Student’s-t test, where t tabulated = 4.303 at 95% confidence level.

**Abbreviations:** SD: Standard Deviation; RSD: Relative Standard Deviation; ANOVA: Analysis of Variance

Table 5: Results for robustness study (no. of determinations=3) of Luliconazole, conc. 300ng/band.

| Sr. No. | Parameters Checked | % RSD of Area | Mean %RSD of Area |
|---------|--------------------|---------------|-------------------|
| 1       | Change in Saturation time |               |                   |
|         | a) At 10 min. (n=3)  | 0.99%         | 1.31%             |
|         | b) At 15 min. (n=3)  | 1.63%         |                   |
| 2       | Change in Mobile Phase Composition |               |                   |
|         | E.A. 0.4 ml (n=3)  | 3.11%         | 2.21%             |
|         | E.A. 0.6 ml (n=3)  | 1.31%         |                   |
| 3       | Change in wavelength (300nm±2) |               |                   |
|         | At 298nm (n=3)      | 0.91%         | 0.95%             |
|         | At 300nm (n=3)      | 1.07%         |                   |
|         | At 302nm (n=3)      | 0.89%         |                   |
| 4       | Change in slit width |               |                   |
|         | 6x0.20mm, micro     | 1.075%        | 1.094%            |
|         | 6x0.45mm, micro     | 1.045%        |                   |
|         | 6x0.40mm, micro     | 1.054%        |                   |
|         | 6x0.10mm, micro     | 1.060%        |                   |
|         | 6x0.30mm, micro (used for analysis) |           | 1.240%            |
| 5       | Change in scan speed |               |                   |
|         | 10mm/sec.           | 1.082%        | 1.12%             |
|         | 40mm/sec.           | 1.052%        |                   |

**Abbreviations:** RSD: Relative Standard Deviation.

Table 6: Result of recovery of Luliconazole (no. of determinations=3 per conc.) in Biological fluid.

| Sr.No. | Conc. (ng/band) | % Recovery (Mean of n=3) | SD  | %RSD |
|--------|-----------------|--------------------------|-----|------|
| 1      | 300             | 100.36                   | 0.69| 0.69 |

**Abbreviations:** SD: Standard Deviation; RSD: Relative Standard Deviation.
**Discussion**

A HPTLC/densitometric method has been developed successfully for the determination of Luliconazole as bulk drug, in marketed formulation and in biological fluid. The estimation of drug was performed on pre-coated silica gel 60 F254 TLC aluminium plates (0.25mm thick) using toluene-methanol-ethyl acetate (6.0:2.5:0.5) as a mobile phase. The densitometric quantification for the drug was carried out at 300nm. The Rf value for Luliconazole was found to be 0.62. The proposed method has been validated for various parameters like linearity, accuracy, precision, sensitivity as per ICH guidelines. The calibration curve was obtained by plotting peak area against concentration. It showed linearity in the concentration range of 100-600ng/band.

Malasia et al. [15] have reported Method Development and Validation of RP HPLC Method for Assay and related Substances of Luliconazole in Topical Dosage form using Inertsil ODS 3V (4.6x 150mm,5μm) by RP-HPLC method. The mobile phase used was ammonium acetate buffer and ACN (60:40). In another study, Desai and Maheshwari[16] have reported U.V. Spectrophotometric method for estimation of Luliconazole in marketed formulation. The LOD and LOQ, 0.38μg/ml and 1.06μg/ml respectively, whereas the HPTLC method showed LOD and LOQ values as 27ng/band and 83ng/band respectively. The proposed HPTLC method is thus found to be more sensitive and economical in comparison to the earlier reported methods [15,16].

The mean recovery for Luliconazole in marketed formulation and biological fluid was found to be 99.91% and 100.36% respectively. As the R values of Luliconazole in bulk, marketed formulation and biological fluid was the same, we could safely conclude that there was no interference of excipients and matrix components. The intraday and inter-day precision results expressed as %RSD were found to be less than 5%. There was no significant difference in %RSD values. F values calculated by one way ANOVA, is less than tabulated at p< 0.01. The Student’s t-test was performed for accuracy. The calculated t value t=1.68 was found to be less than the tabulated value, (t= 4.303) at 95% confidence level, suggesting that the proposed method and the literature method [15] do not differ significantly with respect to accuracy.

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters, and provides an indication of its reliability during normal usage. In robustness study, the RSD percentage was less than 5%. We also propose the current method is convenient for the routine estimation of Luliconazole as a bulk drug in marketed formulation and from biological fluid. Till date no HPTLC method has been reported for quantitative estimation of LCZ except stability indicating method, RP-HPLC and UV spectrophotometric method, so we could develop HPTLC method for analysis of Luliconazole in marketed formulation and biological fluid.

**Conclusion**

The reported HPTLC method has been developed for the identification and quantitation of Luliconazole. It surpasses the earlier methods on the basis of low cost per analysis, faster with satisfactory precision. Method was successfully validated as per ICH guidelines. The validation data, one way ANOVA (F-test) and Student’s t test proves that method is sensitive, specific, precise and statistically significant. It can be conveniently employed for routine quality control analysis of Luliconazole as bulk drug and in marketed formulation without any interference from excipients also in biological fluid. We can further explore this method for various analytical reasons.

**Acknowledgement**

None.

**Conflict of Interest**

None.
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