Development and Validation of a New RP-HPLC Analytical Method for the Simultaneous Determination of Luliconazole and Clobetasol Propionate in Synthetic Mixture

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Authors' contributions

This work was carried out in collaboration with both authors. Author BS designed the study, performed the statistical analysis and wrote the first draft of the manuscript. Author HJ managed the analyses of the study, guided the entire research work and approved the final manuscript.

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ABSTRACT

Aim: To develop new selective and sensitive reverse-phase high-performance liquid chromatography (RP-HPLC) approach for the quantification of antifungal drug Luliconazole integrate with corticosteroid drug Clobetasol Propionate in a synthetic mixture.

Methods: The method was validated to achieve International Conference Harmonization (ICH) requirements. Chromatographic separation was carried out by isocratic technique on a reversed-phase Inertsil C18 column (5 µm, 250mm x 4.6mm i.d with the mixture of Acetonitrile: Water pH adjusted with H3PO4 (60: 40) and UV detection at 264 nm. The compounds were eluted at a flow rate of 1.0 mL/min with an injection volume of 20µL.

Results: The calibration curves were linear (r2 > 0.999) over the concentration range 10-200 µg/mL for Luliconazole and 5-100 µg/mL for Clobetasol Propionate. The average retention times for Luliconazole and Clobetasol Propionate were 3.16 and 6.94 min, respectively. The % RSD for the

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The proposed method was found to be less than %2. The % recovery was found to between 99.22-99.48% for the developed method.

**Conclusion:** The developed method is simple, rapid, precise, and accurate and hence was successfully applied for the determination of Luliconazole and Clobetasol Propionate in a synthetic mixture.

**Keywords:** Antifungal drug; analytical method; glucocorticoids; validation.

### 1. INTRODUCTION

Luliconazole (LLZ), appearing as an antifungal agent, belongs to the azole category. It is likewise acknowledged as (2E)-2-[(4R)-4-(2,4-chlorophenyl)-1,3-dithiolan-2-ylidene]-2-imidazole-1-ylacetonitrile. This agent is hired to deal with skin infections like tinea pedis, jock itch, and ringworm, seborrheic eczema. It interacts with 14-α demethylase, a cytochrome P-450 enzyme that is required for conversion of lanosterol to ergosterol. Luliconazole inhibits ergosterol biosynthesis and alter fungal cell permeability results in cell lysis. Luliconazole is a white powder, poorly water-soluble drug having a molecular weight of 354.267 g/mol [1]. It became authorized via way of means of the FDA (USA) in November 2013 and is advertised below the logo call Luzu. Luliconazole is likewise authorized in Japan.

![Chemical structure of luliconazole](image1)

**Fig. 1. Chemical structure of luliconazole**

Clobetasol Propionate is a selective glucocorticosteroids used to deal with an inflammatory and allergic condition. It is also used to treat Psoriasis, rheumatic problems and skin diseases. Clobetasol propionate (CLP), 21-Chloro-9fluoro-11β-hydroxy-sixteen β-methyl-3,20-dioxopregn-1,4-dien-17-yl propanoate (Fig. 2) is glucocorticoid having a molecular weight of 467.0 g/mol, with chemical formula C_{25}H_{32}ClFO_5 [2]. It is nearly insoluble in water, freely soluble in acetone and dichloromethane, and sparingly soluble in ethanol [3,4]

![Chemical structure of clobetasol propionate](image2)

**Fig. 2. Chemical structure of clobetasol propionate**

### 2. MATERIALS AND METHODS

Luliconazole and Clobetasol Propionate were procured from Kantam pharma, Chhatral, and Farbe Pharma, Ankleshwar respectively. Acetonitrile HPLC grade was purchased from E. Merck Ltd, Mumbai. H_3PO_4 is procured from Antares Chem Private Limited. The analysis of the drug was carried out on a Waters 2646 series HPLC system equipped with a reverse-phase Inertsil C18 column (250x4.6mm, 5μm n particle size) an LC 20 AT isocratic pump, a 20μL injection loop, and an SPD- 20 A Prominence UV- Visible Detector and running on Empower 3 Chromatographic Software version.

#### 2.1 HPLC conditions

The mobile phase made up of ACN and water and the pH were adjusted 3.5 with orthophosphoric acid. The mobile phase was filtered through a 0.45μ membrane filter, and sonication is done for the mobile phase to degases for use. The run time for the standard solution was 10 min. and 1.0 mL/min flow rate is...
maintained for mobile phase. The volume of injection was 20 μL, and the eluent was detected at 264 nm.

2.2 Method

The RP-HPLC method of Luliconazole and Clobetasol Propionate was achieved by isocratic elution technique with UV-VIS Detector. Luliconazole and Clobetasol Propionate was determined at 264 nm respectively with the concentration range of 10-200 μg/mL for Luliconazole and 5-100 μg/mL for Clobetasol Propionate respectively.

2.2.1 Standard stocks solutions of binary mixture

A standard stock solution of LLZ (1000 μg/mL) and CLP (1000 μg/mL) were prepared separately by dissolving 100 mg of drug in 100 mL mobile phase - Acetonitrile: Water pH 3.5(60:40, v/v) pH adjusted with H₃PO₄.

2.2.2 Preparation standard solution of luliconazole and dexamethasone in combination

0.1 mL from working standard stock solutions of LLZ (1000 μg/mL) and 0.05 mL from working standard stock solutions of CLP (1000 μg/mL) were taken in a common volumetric flask diluted up to 10 mL with mobile phase - Acetonitrile: Water pH 3.5 (60:40, v/v) to make final concentration LLZ (10μg/mL) and CLP (5μg/mL).

3. RESULT AND DISCUSSION

The validation of the proposed evolved approach became executed as in keeping with ICH guideline [14]. The method discussed in the present work provides a convenient and accurate way for analysis of LLZ and CLP in the RP-HPLC method. There is no method available for analysis of both the drugs in the combination. So developed RP-HPLC method is useful for analysis of the combination of the drug in synthetic mixture and formulation. Several mobile phase combinations were tried and the mobile phase containing Acetonitrile and Water in ratio 60:40 v/v having pH 3.5(adjusted with H₃PO₄) with the reverse-phase technique was selected due to yielded symmetric and sharp peak with the short run time of 10 min. The wavelengths selected were 264 nm for LLZ and CLP. The plot of area versus respective concentrations of LLZ and CLP were found to be linear in the concentration range of 10-200 μg/mL for LLZ and 5-100 μg/mL for with correlation coefficient 0.999 for both LLZ and CLP as shown in Table 2 and Figs 4-5. Precision was calculated in terms of intraday and interday precision was found to be in the acceptance range (Table 3 and 4). The accuracy of the method was determined by the standard spiking method. The % recovery ranges from 96.80-99.9 % for both the drugs. This method can be successfully used for simultaneous estimation of LLZ and CLP in their combined dosage form.

3.1 Validation of Proposed Method

The validation parameters for the newly developed method are as follow.

3.2 System Suitability Studies parameter

The system suitability was evaluated by five replicate analyses of LLZ and CLP mixture at a concentration of 10 μg/mL of LLZ and 5 μg/mL of CLP. The efficiency of column, resolution factor and peak asymmetry were calculated for the standard solutions.

3.3 Specificity

A blank solution (mobile phase) and Common excipients like Cetosteryl alcohol, liquid paraffin propylene glycol was dispersed in methanol, filtered, and injected. The chromatogram showed no inferring peaks at the retention time of the two drugs which indicates the specificity of the method. This is the final chromatogram of the synthetic mixture using mobile phase Acetonitrile: Water (60:40 v/v) pH 3.5. The retention time for Luliconazole and Clobetasol propionate was found to be 3.16 and 6.94 min.

| Parameters                  | Observed Values | IP’2010 Specification |
|-----------------------------|-----------------|-----------------------|
| Retention Time (min)        | LLZ 3.16        | CLP 6.94              |
| Theoretical plates          | 19871           | 13695                 |
| Asymmetry (10%)             | 1.79            | 1.32                  |
| Resolution                  | 15.45           | >2                    |

Table 1. Observed values for system suitability test
Fig. 3. Chromatogram using mobile phase: Acetonitrile: Water pH3.5 (pH adjust with H₃PO₄) (60: 40 V/V)

3.4 Linearity
The five independent levels of concentration are taken for linearity response for LLZ and CLP. The R² value for the calibration curve of LLZ and CLP was found to be 0.999.

3.5 Precision
3.5.1 Intraday precision
The Intraday precision of the developed method was checked by analysing the sample on the same day for LLZ and CLP.

3.5.2 Interday precision
The Interday precision of the developed method was checked by analysing the sample on a different day for LLZ and CLP.

3.6 Accuracy
The Standard addition method was used for the % recovery study of LLZ and CLP. Percentage recovery indicates that the excipients have no interference.

3.6.1 LOD (limit of detection) and LOQ (limit of quantification)
The LOD and LOQ was determined on basis of signal-to-noise ratio. The LOD and LOQ are estimated from the set of 5 calibration curves used to determine method linearity.

LOD = 3.3σ/S.
LOQ = 10σ/S
Where, S = Slope of the calibration curve

3.6.2 Robustness and ruggedness
The robustness and ruggedness study of the developed method were done by doing a small change in method condition for both the drug LLZ and CLP.

### Table 2. Calibration data for LLZ and CLP

| Sr. No | Concentration (µg/mL) | Peak area ± SD LLZ | Peak area ± SD CLP |
|--------|-----------------------|---------------------|---------------------|
|        | LLZ                  |                     |                     |
| 1      | 10                    | 1029236 ± 712.479   | 789624 ± 750.891    |
| 2      | 50                    | 1223654 ± 820.973   | 1025896 ± 984.369   |
| 3      | 100                   | 1452101 ± 747.143   | 1284998 ± 657.219   |
| 4      | 150                   | 1649998 ± 817.292   | 1522764 ± 867.467   |
| 5      | 200                   | 1878112 ± 758.679   | 1799989 ± 914.255   |

|        | CLP                  |                     |                     |
| 1      | 5                     |                      |                     |
| 2      | 25                    |                      |                     |
| 3      | 50                    |                      |                     |
| 4      | 75                    |                      |                     |
| 5      | 100                   |                      |                     |

### Table 3. Intraday precision data for LLZ AND CLP

| Concentration (µg/mL) | Peak area ± SD LLZ | % RSD | Peak area ± SD CLP | % RSD |
|-----------------------|--------------------|-------|--------------------|-------|
| LLZ                   |                    |       | CLP                |       |
| 10                    | 1029663 ± 874.654  | 0.084 | 789201 ± 815.427   | 0.103 |
| 50                    | 1223469 ± 919.700  | 0.075 | 1025567 ± 1107.24  | 0.107 |
| 100                   | 1453941 ± 534.430  | 0.036 | 1284464 ± 774.012  | 0.060 |
Fig. 4. Calibration curve of standard CLP

Fig. 5. Calibration curve of standard LLZ

Table 4. Interday precision data for LLZ AND CLP

| Concentration (µg/mL) | Peak area* ± SD LLZ | % RSD | Peak area* ± SD CLP | % RSD |
|----------------------|---------------------|-------|---------------------|-------|
| LLZ                  | CLP                 |       |                     |       |
| 10                   | 5                   | 1029896 ± 709.10 | 0.068 | 789201 ± 843.76 | 0.106 |
| 50                   | 25                  | 1223896 ± 1010.38 | 0.082 | 1025567 ± 1009.8 | 0.098 |
| 100                  | 50                  | 1452963 ± 838.073 | 0.057 | 1284464 ± 618.64 | 0.048 |

Table 5. Recovery study

| Concentration (µg/mL) formulation | Spiked level (µg/mL) | % Recovery ± SD |
|-----------------------------------|----------------------|-----------------|
| LLZ                              | CLP                  | LLZ             | CLP             |
| 20                                | 10                   | 0%              | -               | 99.00±0.079 | 99.80±0.026 |
| 20                                | 10                   | 80%             | 16              | 98.88±0.511 | 98.83±0.526 |
| 20                                | 10                   | 100%            | 20              | 99.25±0.017 | 99.85±0.045 |
| 20                                | 10                   | 120%            | 24              | 99.77±0.053 | 99.45±0.125 |

Table 6. LOD and LOQ data of LLZ and CLP

| Parameter | LLZ (10 µg/mL) | CLP (5 µg/mL) |
|-----------|----------------|---------------|
| LOD (µg/mL) | 0.032         | 0.029         |
| LOQ (µg/mL) | 0.099         | 0.090         |

3.7 Assay

The proposed validated methods were successful applied to determine Luliconazole and Clobetasol Propionate in a synthetic mixture. The equivalent weight of Luliconazole and Dexamethasone in a synthetic mixture was 200mg and 100 mg respectively.
Table 7. Ruggedness and robustness study

| No | Factor                              | Level  | Peak area* ± SD  | %RSD  | R_t ± SD  | %RSD  |
|----|-------------------------------------|--------|------------------|-------|-----------|-------|
| 0. | Mobile phase: Mobile phase: Acetonitrile: Water pH 3.5 (60:40 v/v) |        |                  |       |           |       |
| 1. | Change in the Flow Rate ± 0.1 mL/min | 0.9 mL/min | 1029663 ± 817.31 | 0.079 | 3.15 ± 0.015 | 0.478 |
|    |                                     | 1.1 mL/min | 1029986 ± 712.95 | 0.069 | 3.16 ± 0.013 | 0.438 |
| 2. | Change in Mobile phase composition ± 5% | 43: 57 | 1029986 ± 844.88 | 0.082 | 3.11 ± 0.016 | 0.519 |
|    |                                     | 37: 63  | 1057896 ± 955.964 | 0.090 | 3.14 ± 0.019 | 0.617 |
| 3. | Change in Column                     | Phenomenex | 1028896 ± 622.74 | 0.060 | 3.16 ± 0.019 | 0.624 |
|    |                                     | Luna    | 1029999 ± 1005.48 | 0.097 | 3.14 ± 0.014 | 0.469 |

Ruggedness and Robustness data of CLP (5 μg/mL)

| No | Factor                      | Level  | Peak area* ± SD  | %RSD  | R_t ± SD  | %RSD  |
|----|------------------------------|--------|------------------|-------|-----------|-------|
| 0. | Mobile phase: Mobile phase: Acetonitrile: Water pH 3.5 |        |                  |       |           |       |
| 1. | Change in the Flow Rate ± 0.1 mL/min | 0.9 mL/min | 789264 ± 965.67 | 0.122 | 6.91 ± 0.039 | 0.566 |
|    |                                     | 1.1 mL/min | 789385 ± 871.91 | 0.110 | 6.93 ± 0.015 | 0.216 |
| 2. | Change in Mobile phase composition ± 5% | 43: 57 | 789258 ± 670.60 | 0.085 | 6.92 ± 0.019 | 0.283 |
|    |                                     | 37: 63  | 789235 ± 996.74 | 0.126 | 6.93 ± 0.012 | 0.185 |
| 3. | Change in Column                | Phenomenex | 789258 ± 974.07 | 0.123 | 6.92 ± 0.035 | 0.505 |
|    |                                     | Luna    | 789235 ± 811.27 | 0.102 | 6.94 ± 0.016 | 0.243 |

Table 8. Estimation of LLZ and CLP in synthetic mixture

| Sr. No. | Drug name | Formulation (Synthetic mixture μg/mL) (μg/mL) | %Assay* |
|---------|-----------|-----------------------------------------------|---------|
| 1       | LLZ       | 10                                           | 99.96±0.011 |
| 2       | CLP       | 5                                            | 99.58±0.009 |

Where *(n=3)
3.8 Result

| Parameters                        | LLZ          | CLP          |
|-----------------------------------|--------------|--------------|
| Concentration range (µg/mL)       | 10-200       | 5-100        |
| Regression equation               | Y=4420.8x + 995703 | Y=10482x+749998 |
| Correlation Coefficient(R²)       | 0.999        | 0.999        |
| Accuracy (%Recovery) (n=3)        | 99.22        | 99.48        |
| Intra-day Precision (%RSD) (n=3)  | 0.0367-0.0849 | 0.060-0.106  |
| Inter-day Precision (%RSD) (n=3)  | 0.0576-0.0826 | 0.048-0.106  |
| LOD (µg/mL)                       | 0.032        | 0.029        |
| LOQ (µg/mL)                       | 0.099        | 0.090        |
| Robustness and Ruggedness         | 0.060-0.097  | 0.08-0.216   |
| %Assay                            | 99.96        | 99.56        |

4. CONCLUSION

The developed RP-HPLC method is simple, specific, accurate, and precise and is validated for simultaneous estimation of Luliconazole and Clobetasol Propionate as per ICH Q2R1[14] guidelines. Inertsil C18 column (250 mm x 4.6mm, 5µm) was used for the chromatographic separation using ACN: Water (60:40 v/v) pH 3.5 as mobile phase at 264 nm with flow rate1.0 mL/min and injection volume 20 µL. The plot of area versus respective concentration of Luliconazole and Clobetasol Propionate was found to be linear in the concentration range of 10-200 µg/mL for Luliconazole and 5-100 µg/mL for Clobetasol Propionate with R² =0.999 for both LLZ and CLP. Precision was calculated as interday and intraday variations and % RSD was found to be less than 2% for both the drugs. Mean % recovery for Luliconazole and Clobetasol Propionate was found to be 99.91 % and 99.06 % respectively. % Assay for Luliconazole and Clobetasol Propionate was found to be 99.22 and 99.48% indicates that method is accurate. System suitability test reveals that all system suitability parameters comply with standard values.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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