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UV AND RP -HPLC METHOD DEVELOPMENT AND VALIDATION OF LEVOKETOCONAZOLE

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ABSTRACT:
Objective: This study aimed to develop a precise and reliable method for the estimation of levoketoconazole in bulk drug and pharmaceutical dosage forms using UV-visible spectrophotometry and RP-HPLC.
Method: UV-visible spectrophotometric determination was performed using an Elico double-beam SL 210 UV-visible spectrophotometer with a deuterium lamp at λmax 296 nm using methanol as the medium. The UV-visible spectrophotometric and HPLC methods were linear over concentration ranges of 50–250 µg/ml and 10–100 µg/ml, respectively. HPLC analysis was performed using a C18 column (1.7 micron) with a photodiode array detector and a quaternary pump at a flow rate of 1 ml/min. The run pressure was 2140 psi. Acetonitrile:water (20:80) was used as the mobile phase, and the effluent was monitored at 296 nm. Both methods were validated for linearity, precision, accuracy, robustness, ruggedness, detection and quantification limits, and formulation analysis according to ICH guidelines.
Results: The retention time was 3.8 minutes, and the RSD for precision studies and recovery analysis of the UV and HPLC methods was less than 1%. The reported recovery values were 99.5–100.8% for HPLC and 70–86% for UV.
Conclusion: Both methods were validated according to ICH guidelines. The developed methods were applied for the estimation of levoketoconazole in bulk drug and pharmaceutical dosage forms.

KEYWORDS: Levoketoconazole; Method development and validation; ICH guideline; UV-visible method; RP-HPLC; Validation;Public Health, Global Health.

INTRODUCTION

The 2S,4R stereoisomer of ketoconazole, levoketoconazole (Recorlev), is an oral adrenal steroidogenesis inhibitor developed for the treatment of endogenous Cushing's syndrome (CS) [1]. It is a white crystalline powder and an imidazole derivative. The chemical name of levoketoconazole is 1-[4-[4-[[(2S,4R)-2-(2,4-dichlorophenyl)-2-(imidazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy]phenyl] piperazin-1-yl] ethenone, with a molecular formula of C26H28Cl2N4O4 and a molecular weight of 531.4 g/mol.

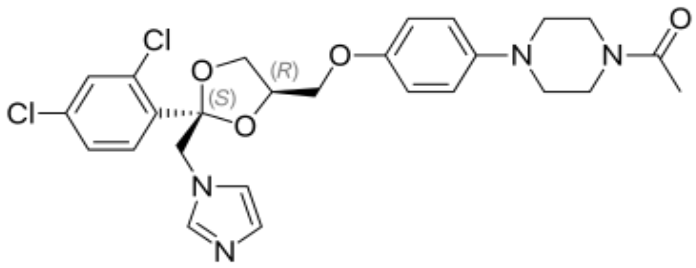


Figure 1. Chemical structure of levoketoconazole.

A New Drug Application was approved by the US Food and Drug Administration (FDA) on December 30, 2021, for the treatment of endogenous hypercortisolemia in people with Cushing's syndrome for whom surgery is not an option or has not proved curative. Chronic exposure to excessive cortisol results in a rare and serious endocrine condition known as endogenous Cushing's syndrome. The main goal of treatment is to normalize cortisol production or activity [2].As with any medication, levoketoconazole can have adverse effects and should be used under medical supervision. Commonly reported effects include nausea, headache, and anxiety.
A review of the literature identified limited analytical information for levoketoconazole and supported the need for straightforward spectrophotometric and chromatographic methods [3–6]. The present study therefore

developed and validated UV-visible spectrophotometric and RP-HPLC methods for the estimation of levoketoconazole in API and tablet dosage forms.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

The chemicals used were methanol (AR grade), acetonitrile (AR grade), and Millipore water (HPLC grade), with levoketoconazole working reference standard.

2.2 Selection of Drug

Levoketoconazole API was purchased from Sim Son Pharma Limited, Mumbai.

2.3 Instrument Specifications

UV analysis was performed using a Shimadzu UV-1800 UV-visible spectrophotometer with a deuterium lamp and SpectraTreat software. HPLC analysis was conducted using a Shimadzu HPLC-LC 20AT SRCOP/IC/138 system equipped with a PDA detector at 296 nm, a Waters Reliant C18 column (250 × 6 mm, 1.7 microns), and a Quaternary pump.

2.4 Selection of Solvent for the UV Method

Levoketoconazole was soluble in methanol, and the UV-visible spectrophotometric method was performed using methanol as the solvent. The absorbance of the drug was high when methanol was used.

2.5 Selection of Solvent for the HPLC Method

Levoketoconazole was soluble in acetonitrile, and HPLC analysis was performed using acetonitrile. The absorbance of the drug was high when acetonitrile was used.

2.6 Spectrophotometric and Chromatographic Conditions

Parameter	Condition
UV diluent	Methanol
Detection wavelength	296 nm
HPLC column	Waters Reliant C18 (250 × 6 mm, 1.7 microns)
Mobile phase	Acetonitrile:water (20:80)
Flow rate	1 ml/min
Pressure	2140 psi
Injection volume	20 µl
Run time	10 min
Detection	296 nm

2.7 Preparation of Standard Solutions

2.7.1 HPLC Method

Levoketoconazole (10 mg) was accurately weighed and transferred to a 10 ml volumetric flask. Approximately 5 ml of acetonitrile was added and the mixture was sonicated to dissolve the drug. The volume was made up with the same solvent to obtain a stock solution of 1000 µg/ml. Serial dilution was used to obtain 1, 2, 4, 8, 20, 40, and 100 µg/ml.

2.7.2 UV Method

A 100 ml volumetric flask was filled with 100 mg of accurately weighed levoketoconazole and methanol to obtain a main stock solution of 1000 µg/ml. One milliliter of the stock solution was pipetted and diluted with methanol to obtain a working solution of 100 µg/ml in a 10 ml volumetric flask.

2.8 Preparation of Sample Solutions

2.8.1 HPLC Method

Two levoketoconazole-coated tablets were taken and pulverized. Powder equivalent to 10 mg of levoketoconazole was accurately weighed and transferred to a 10 ml volumetric flask. Five milliliters of acetonitrile was added and the mixture was sonicated for 10 minutes with periodic shaking. Acetonitrile was added to volume to obtain a 1000 µg/ml solution. The solution was filtered through a membrane filter and appropriately diluted with acetonitrile to obtain 50 µg/ml.

2.8.2 UV Method

Ten levoketoconazole tablets were taken and pulverized. Powder equivalent to 10 mg of levoketoconazole was accurately weighed and transferred to a 100 ml volumetric flask. Fifty milliliters of methanol was added and the mixture was sonicated for 10 minutes with periodic shaking. The volume was made up to 100 ml with methanol to obtain a 1000 µg/ml solution. The solution was filtered through a membrane filter. One milliliter was transferred and diluted with methanol to obtain 100 µg/ml in a 10 ml volumetric flask.

2.9 Method Validation

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2 R1 criteria for validation of analytical procedures were followed.

2.9.1 Linearity

Linearity is the ability of an analytical procedure to obtain test results that are directly proportional to the concentration of analyte in the sample within a specified range [7]. For the UV method, five standard solutions

between 50 and 250 µg/ml were examined at 296 nm. For HPLC, standard solutions in the 10–100 µg/ml range were examined. Calibration curves were prepared by plotting average absorbance or peak area against concentration. The study was conducted three times for both methods.

2.9.2 Precision

Precision describes the closeness of agreement among a series of measurements obtained from multiple samples of the same homogeneous sample under prescribed conditions [8]. Intraday precision was evaluated using six duplicate standard solutions on the same day, while interday precision was evaluated over three days (50 µg/ml for HPLC and 150 µg/ml for UV).

2.9.3 Accuracy and % Recovery

For HPLC, tablet powder equivalent to 10 mg of levoketoconazole was transferred to a 10 ml volumetric flask and dissolved in acetonitrile. Aliquots of 2, 3, and 4 ml were transferred into 10 ml volumetric flasks containing levoketoconazole standard solution (20 µg/ml), and acetonitrile was added to obtain final concentrations of 40, 50, and 60 µg/ml.

For UV, powder equivalent to 100 mg of levoketoconazole was added to a 100 ml volumetric flask and dissolved in methanol. Aliquots of 1 ml were transferred into 10 ml volumetric flasks with 0.5, 1, and 1.5 ml of levoketoconazole standard solution to obtain final concentrations of 150, 200, and 250 µg/ml. Each solution was prepared in triplicate and analyzed.

2.9.4 Limit of Detection and Limit of Quantification

The LOD and LOQ were determined using the standard deviation of the response and the slope of the calibration curve [9].

$LOD = 3.3\sigma/S$

$LOQ = 10\sigma/S$

2.9.5 Robustness

Robustness indicates the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters [10]. Three duplicates of each sample were used at 50 µg/ml for both UV and HPLC.

2.9.6 Ruggedness

Ruggedness was evaluated using the same sample under different analysts, laboratories, or instruments [11].

2.9.7 Assay Method

Ten tablets (150 mg) were accurately weighed and crushed. A 100 µg/ml standard and sample solution were compared for the UV method. Similarly, a 50 µg/ml standard and sample solution were compared for the HPLC method. The percentage purity of the sample and standard was determined using both methods.

3. RESULTS AND DISCUSSION

3.1 UV Method Development

The UV-visible spectrum of levoketoconazole in methanol showed maximum absorption at 296 nm. This wavelength was therefore selected for spectrophotometric estimation.

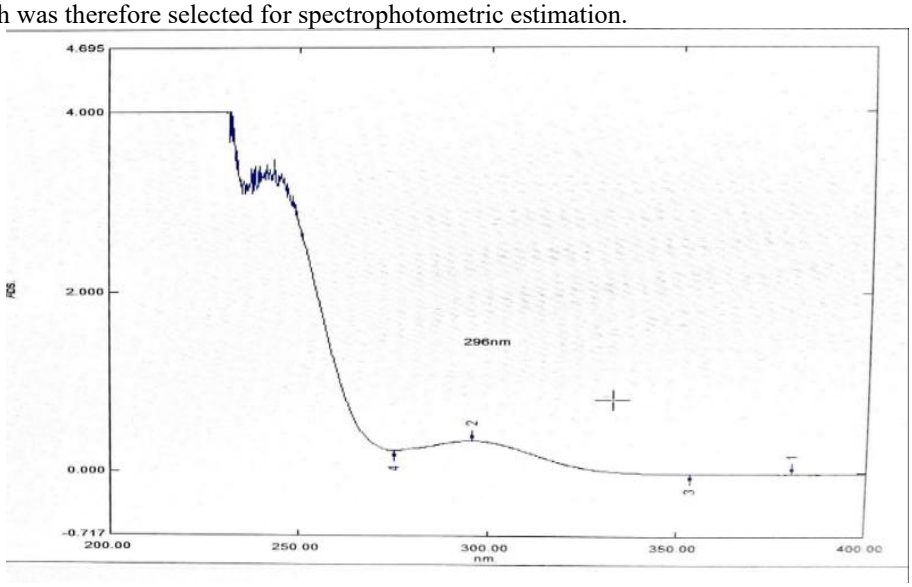
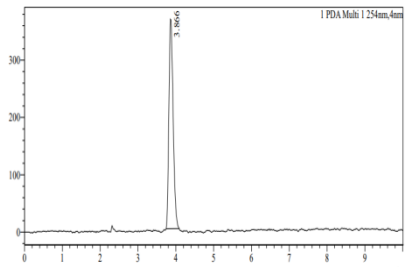
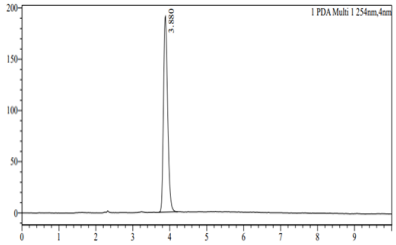


Figure 2. UV-visible spectrum of levoketoconazole in methanol.

3.2 RP-HPLC Method Development

The optimized chromatographic conditions used acetonitrile:water (20:80) as the mobile phase with a flow rate of 1 ml/min, a C18 column, a 20 µl injection volume, a 10-minute run time, and detection at 296 nm. The retention time of levoketoconazole was 3.8 minutes.



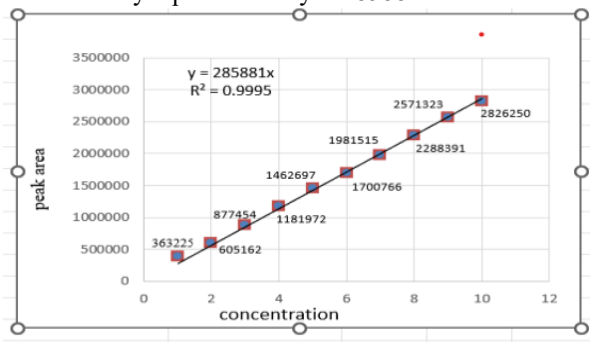


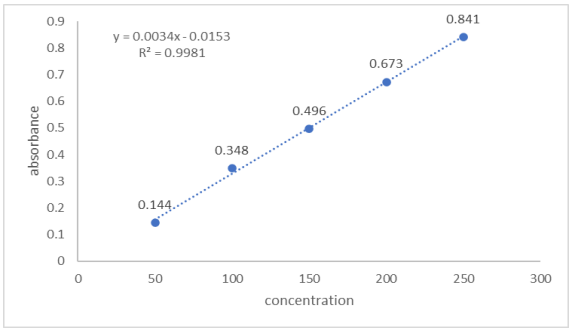
(A) Levoketoconazole standard chromatogram chromatogram **(B) Levoketoconazole sample**

Figure 3. Representative RP-HPLC chromatograms of (A) levoketoconazole standard and (B) levoketoconazole sample.

3.3 Linearity

Using the UV technique, linearity was established by plotting absorbance against concentration. Over 50–250 µg/ml, the regression coefficient was 0.9981 and the regression equation was y = 0.0034x + 0.0153. For HPLC, peak area was plotted against concentration over 10–100 µg/ml. The regression coefficient was 0.9995 and the reported linearity equation was y = 285881x.





(A) RP-HPLC calibration curve **(B) UV calibration curve**

Figure 4. Calibration curves of levoketoconazole obtained by (A) RP-HPLC) and (B) UV-visible spectrophotometry.

3.4 Precision

The percentage RSD for the precision analysis was within the reported acceptance criterion of <2% RSD. The results are presented in Tables 2–5.

Inter-day precision: UV method

Table 2. Inter-day precision analysis data of levoketoconazole for UV

Absorbance	Standard deviation	Mean	%RSD
0.479	0.00081240	0.48083	0.16%
0.479	0.00081240	-	0.16%
0.481	0.00091045	-	0.18%
0.481	0.00091045	-	0.18%
0.482	0.00089423	-	0.18%
0.482	0.00089445	-	0.18%

Inter-day precision: HPLC method

Table 3. Inter-day precision analysis data of levoketoconazole for HPLC.

Peak area	Mean	Standard deviation	%RSD
1470337	-	14207.97	0.94%
1502107	-	6359.824	0.42%
1487886	-	11589.54	0.77%

1528022	-	7645.116	0.50%
1519202	-	1099.251	0.07%
1504565	-	233.8927	0.01%

Intra-day precision: UV method

Table 4. Intra - day precision analysis data of levoketoconazole for UV.

Day	Readings	Mean	Standard deviation	%RSD
Day 1	0.478, 0.479, 0.481	0.479	0.000707	0.14%
Day 2	0.473, 0.475, 0.476	0.474	0.00070	0.14%
Day 3	0.472, 0.474, 0.474	0.473	0.0007071	0.14%

Intra-day precision: HPLC method

Table 5. Intra-day precision analysis data of levoketoconazole for HPLC.

Day	Peak area	Mean	Standard deviation	%RSD
Day 1	1519202 ± 3	-	300.87393	0.19%
Day 2	1512266 ± 3	1514947	1895.7532	0.12%
Day 3	1513373 ± 3	-	1112.9865	0.07%

3.5 Accuracy and Recovery

Recovery experiments were used to determine the accuracy of the HPLC and UV-visible methods. Three concentration levels were used for UV (150, 200, and 250%) and HPLC (80, 100, and 120%). Each study was conducted in triplicate. The reported RSD values were below the stated theoretical limit of <2% RSD.

Table 6. Recovery analysis for levoketoconazole by UV and HPLC methods.

Method	Concentration (µg/ml)	Sample added (µg/ml)	Standard added (µg/ml)	% Recovery	%RSD
UV	150	100	50	70%	0.14%
UV	200	100	100	77%	0.14%
UV	250	100	150	86%	0.14%
HPLC	40	20	20	100%	0.02%
HPLC	50	30	20	99.5%	0.21%
HPLC	100	40	20	100.8%	0.04%

3.6 Limit of Detection and Limit of Quantification

Using the suggested UV technique, the LOD and LOQ for levoketoconazole were 34.3409 µg/ml and 104.0402 µg/ml, respectively. The suggested HPLC approach revealed LOD and LOQ values of 0.125 µg/ml and 100 µg/ml, respectively.

Table 7. Reported LOD and LOQ values of levoketoconazole.

Method	LOD (µg/ml)	LOQ (µg/ml)
UV	34.3409	104.0402
HPLC	0.125	100

3.7 Robustness

The robustness of the HPLC method to temperature changes and variations in mobile-phase ratio was examined. The results showed that temperature changes influenced retention time, while the peak remained symmetrical and repeatability was maintained. For the UV method, the effect of wavelength variation was examined and repeatability was unaffected.

HPLC robustness

Table 8. Results obtained for robustness study of the HPLC method.

Parameter	Condition	RT	Area	Peak shape
Change in temperature	+2˚C	3.859	1515817	Symmetrical peak
Change in temperature	-2˚C	3.870	1493257	Symmetrical peak
Change in mobile-phase ratio	18:82 (+2˚C)	3.792	1510360	Symmetrical peak
Change in mobile-phase ratio	22:78 (-2˚C)	4.007	1503276	Symmetrical peak

3.7 Robustness (continued)
UV robustness

Table 9. Results obtained for robustness study of the UV method.

Parameter	Condition	Absorbance (nm)	% of change
Change in wavelength	297 nm	0.157	0.001
Change in wavelength	298 nm	0.158	0.002

The %RSD values for the robustness parameters were reported to be below the acceptable theoretical limit of <2% RSD. The proposed UV and HPLC methods were therefore evaluated for robustness under the stated conditions.

3.8 Ruggedness
The standard solutions of levoketoconazole were analyzed for ruggedness. The findings reported in the manuscript demonstrated no statistically significant differences across laboratories, analysts, or instruments. The stated acceptance limit was ±1 for both methods, and the methods were considered rugged.

3.9 Determination of Active Ingredient in Tablet Dosage Form (Assay)
Under similar experimental conditions, the samples were analyzed using both methods. The results indicated that the drug content was within the limits prescribed by the stated criteria.

Table 10. Formulation analysis results.

S. No.	Tablet	Dose	Sample concentration	Amount present	% Purity
1	Levoketoconazole (UV)	150 mg	100 µg/ml	0.149136 mg/ml	99.42%
2	Levoketoconazole (HPLC)	150 mg	50 µg/ml	146.946 mg/ml	97.96%

The formulation results obtained using UV and HPLC were compared statistically. The manuscript reports that the Fcal value was less than the Ftab value, indicating acceptance of the null hypothesis and no discernible difference between the sample concentration estimated by either method. The results supported the selectivity of both approaches for formulation estimation.

4. CONCLUSION
Using UV and HPLC techniques, an accurate, affordable, accessible, dependable, and repeatable approach for estimating levoketoconazole in bulk and tablet dosage forms was developed and validated in accordance with ICH recommendations. The broad range of linearity and the use of affordable, readily accessible mobile phases and dilution fluids provide potential for application of the proposed levoketoconazole estimation techniques. The proposed UV and HPLC procedures were considered reliable according to the reported ICH-based assessment, with RSD values for the validation parameters stated to be less than 2%.

CREDIT AUTHOR STATEMENT
All the authors equally participated in the conception and design of the study, data acquisition, data analysis, and data interpretation. They actively participated in drafting the article and revising it critically for important intellectual content and approved the final version to be submitted.

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COMPETING INTERESTS
The authors have no relevant financial or non-financial interests to disclose.

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