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Method Development and Validation of RP HPLC Method for Assay and related Substances of Luliconazole in Topical Dosage form

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Abstract

A simple, specific, accurate, precise , rapid, robust and selective stability indicating reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for assay and related substances and validated for quantification of antifungal drug with its excipients in its topical dosage form. Forced degradation study is done to determine stability of the product. The mobile phase has been used for separation consisting of ammonium acetate buffer: ACN (60:40). Column used was inertsil ODS 3V (4.6*150 mm, 5µm) with flow rate of 1.0 ml/min. Detection wavelength for luliconazole was 294 nm and for methyl paraben was 254nm. The method has been linear for both compounds at 50-150% range with r^2 0.998 and 0.999 respectively. Luliconazole and methyl paraben has showed 98-102% recovery. The method have been robust under various variation with flow rate, detection wavelength and column oven temperature. The drug was exposed to stress conditions. Method resolves all degraded product as compared to luliconazole. Developed method can be used routinely for estimation of drug luliconzole with its excipient methyl paraben in dosage form and stability sample.

Keywords: Luliconazole, Methyl paraben, RP-HPLC, Stability, Antifungal, Forced degradation

Introduction[1,2,3,4,5]

2-[(2E, 4R)-4-(2,4- dichlorophenyl)-1,3- dithiolan-2 ylidene]-2 – (1,H imidazole – 1- yl) is chemical name of luliconazole. It is yellowish powder. It is soluble in acetonitrile, di methyl formamide, di methyl sulphoxide. It is an imidazole antifungal agent. Luliconazole Cream is indicated for the topical treatment of interdigital tineapedis, tineacruris, and tineacorporis.

Methyl paraben is a methyl ester of *p*-hydroxybenzoic acid. It is a stable, non-volatile compound used as an antimicrobial preservative in foods, drugs and cosmetics for over 50 years. Methyl paraben is readily and completely absorbed through the skin and from the gastrointestinal tract. A review of literature is compassed to discover the reported method of luliconazole with its excipients (methyl paraben). There's not a single method reported for simultaneous estimation. Few methods are reported for luliconazole. LC-MS method and HPLC methods has been reported.

Experimental

Apparatus: Waters HPLC e2695 separation module equipped with UV- visible detector and diode array detector, with empower software. Column used was Inertsil ODS 3V, 150 x 4.6; 5µm, analytical balance (Startorious), ultrasonic bath (Oscar ultrasonic microclean-109)

Reagents and materials

Working standard grade of luliconazole and methyl paraben was supplied by Cadila Pharmaceuticals (Dholka, India), Luli RX, marketed formulation with label claim 1% luliconazole, 1.4% methyl paraben and placebo was manufactured and provided by Cadila Pharmaceuticals. (Dholka, India). Acetonitrile of HPLC grade, di methyl formamide, 0.45 mm PVDF filters were also supplied by Cadila Pharmaceutical Ltd.

Chromatographic conditions

1. Instrument: High performance liquid chromatography
2. Column: Inertsil ODS 3-V (150x 4.6mm), 5µm
3. Flow rate: 1ml/min
4. Column oven temperature: 30ºC
5. Run time: 25 min
6. Detector: 294nm for luliconazole 254nm for methyl paraben

Table 1: Gradient program

Time (min)	Mobile phase (A) Amm. Acetate buffer	Mobile Phase (B) ACN
0	60	40
10	10	90
18	60	40
25	60	40

Preparation of Standard solution

Stock solution: Weigh 10 mg of luliconazole, 14 mg methyl paraben, reference standard, transfer it to 100 ml volumetric flask. Add 10 ml dimethyl formamide. Sonicate to dissolve. Add 60 ml acetonitrile and sonicate for 3 minute. Make the volume with acetonitrile upto the mark and filter with 0.45 µm PVDF filters [**Use for RS (500 ppm**)]

Standard for solution luliconazole: Pipette out 5 ml from stock solution in 50 ml volumetric flask. Make the volume with diluent upto the mark and filter with 0.45 µm PVDF filters (**Use for AS**)

Standard solution for Methyl paraben: Pipette out 5ml from stock solution in 100 ml volumetric flask and make up with diluent upto the mark.

Diluent: Prepare a mixture of acetonitrile and milli Q water in ratio of 50:50 (v/v) respectively and mix well.

Ammonium acetate buffer Weigh 0.777 gm 0.01M of ammonium acetate in 1000 ml Milli Q water. Mix well and sonicate to degas the solution.

Sample Preparation

Stock Solution

Sample for RS: Weigh 1gm of sample in 20 ml volumetric flask. Add 5 ml dimethyl formamide, shake well to disperse cream. Add about 5ml of acetonitrile and sonicate for 3 minute. Make the volume with acetonitrile upto the mark. Filter this solution from 0.45 micron PVDF filter. Discard first 5 ml.

Sample for AS: Pipette out 2ml from filtrate in 20 ml volumetric flask. Make the volume with diluent upto the mark. (Use for AS)

System suitability Test: System suitability test was performed before validation run using five replicate injections of a standard solution. Theoretical plates, tailing factor and resolution were determined.

Table 2: Procedure for stress condition

Area of stress	Degradation condition	After degradation treatment
Heat	a) Heat the sample for 1 hour at 60ºC b) Heat the sample for 1hr at 80ºC	Cool down at room temperature then follow as per methodology
Acid	Add 1ml 0.1 N Hcl, heat for an hour at 60ºC	Cool down at room temp, neutralize with 0.1 N NaOH, then follow as per methodology
Base	Add 1ml 0.05 N Hcl, heat for an hr at 60ºC	Cool down at room temp, neutralize with 0.05 N HCl, then follow as per methodology
Oxidation	Add 3ml 3% H_2O_2 heat for an hour at 60ºC	Cool down at room temp, then follow as per methodology

Method validation for assay

Accuracy (% recovery): The accuracy of method was determined by calculating recoveries by spiking method. Known amount of standard solutions of 50%, 100% and 150% were made for HPLC method. The amount was estimated by the regression equation of the calibration curve.

Method precision: The precision of the instrument was checked by repeatedly injecting (n=5) standard solutions of 150 µg/ml for HPLC method.

Limit of Detection and Quantitation: The limit of quantitation and limit of detection of drug was calculated using the following equation as per International Conference on harmonization (ICH) guideline.

LOD = 3.3* (X/S) LOQ = 10 * (X/S)

Where X=the standard deviation of the response and S= the standard deviation of y-intercept of regression lines.

Robustness: Prepare the standard solution as per methodology and analysed by varying following chromatographic conditions.

- **Variation in flow rate**: ±0.1ml/min (0.9ml/min and 1.1ml/min)
- **Variation in column temperature**: ± 5ºC (25 ºC and 35 ºC)
- **Variation in wavelength**: ± 2 nm (292 nm and 296 nm for luliconazole, 252 and 256 for methyl paraben and 278nm and 282 nm for BHT)
- **Variation in mobile phase ratio**: For BHT (buffer: ACN , 10:90 and 20:80)

The robustness was performed by changing the flow from 1ml/min to ±, by changing temperature from 30°C to ± 5°C and by changing wavelength by ± 2°C.

Analysis of Luliconazole and methyl paraben in its topical dosage form: Cream containing 1% luliconazole and 1.4% in methyl paraben were selected for the study. The response of dosage form was measured at 294 nm, 254 nm respectively. The amount of sample solution was determined by fitting the peak area into the regression equation of HPLC.

Result and Discussion

HPLC Method: To optimize the HPLC parameters several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for simultaneous estimation of luliconazole and methyl paraben was obtained in mobile phase 0.1 M ammonium acetate buffer and ACN (60:40) ratio. To obtain better result quantification was achieved by UV detection at 294 nm based peak area. A complete resolution of peaks with clear baseline separation was obtained.

System suitability test: The system suitability test of chromatographic system was performed before validation run. The system suitability test is integrated part of analytical method and it ascertains the suitability and effectiveness of the operating system. The approximate results are mentioned in Table1.

Degradation behavior: The result of forced degradation are summarized in the Table 3.

Table 3: Results of forced degradation studies

Condition		Weight taken in mg	Area	% Assay	% Degradation
As such	-	1135.76	3148214	100.1	nil
Acid	1ml,0.1Hcl 45ºC for 1 hr	1009.32	2079728	74.3	25.7
Base	1ml,0.05N NaOH 45ºC for 1 hr	1057.37	2300666	78.5	21.5
Hydrolysis	5ml water, 1 hour	1014.58	2356470	86	13
Oxidative	3 ml, 3% H_2O_2	957.2	1870148	70.7	30
Thermal	80ºC for 2 hour	1019.03	2456470	87	14

The results obtained for forced degradation were within 10-30% for acid, base condition, main peak was free from interference from other degradation peaks, hence method is specific for stability indicating assay method. No degradation has been observed in oxidative, thermal and hydrolysis degradation.

Validation for proposed method: Both luliconazole and methyl paraben has shown linear response in range of 50-150% for HPLC. The dosage form recovered 98%- 102% by HPLC (Table5). The total impurity for drug luliconazole was found to be 0.68%. The precision of luliconazole and methyl paraben was within the limit and the % RSD was 0.6% and 0.5 % respectively. The method found to be robust as the plate count were more than 2000, tailing factor was found less than 2 and % RSD was also less than 2.

Assay of the cream: The proposed validated methods were successful applied to determine luliconazole and methyl paraben in topical dosage form (**LULI RX**, 1% luliconazole and 1.4% methyl paraben)

Related substance study: The test was performed on "LULI RX" to determine the effects of impurity on the product. It was found that total impurity percentage was 0.68% which is within the limits. There was no interference of any impurity on main peak.

Table 4: Summary of validation parameters by HPLC method for assay and related substance

S. No.	Parameters	Assay of Luliconazole	Assay of methyl parben	Related substance Luliconazole
1.	Detection wavelength(nm)	294 nm	254 nm	294 nm
2	Retention time (min)	8.34	3.82	8.37
3	Coefficient correlation (r^2)	0.9998	0.9999	0.9905
4	Intercept	63883.986	1832.159	-52416.41
5	Slope	29205.37	8599.272	3597.62
6	Assay	102.7	101.3	_
7	% recovery	99.45	101.38	99.07
8	Precision	0.6	0.5	
9	Limit of detection	_	_	0.032
10	Limit of quantitation	_	_	0.485
11	**System suitability parameters**			
A	Retention time (min)	8.341	3.833	8.29
B	Theoretical plate	46420	4380	41412
C	Tailing factor	1.1	1.2	1.1

Table 5: Estimation of Luliconazole and methyl paraben in Cream by HPLC

Formulation	Label claim		Luliconazole	Methyl paraben
Luli Rx (Cadila Healthcare Ltd)	1%	1.4%	102.7 %	101.3%

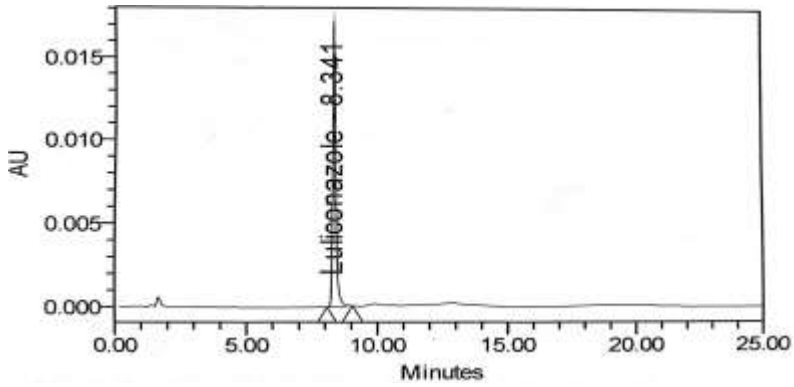


Fig. 1: Chromatogram of luliconazole

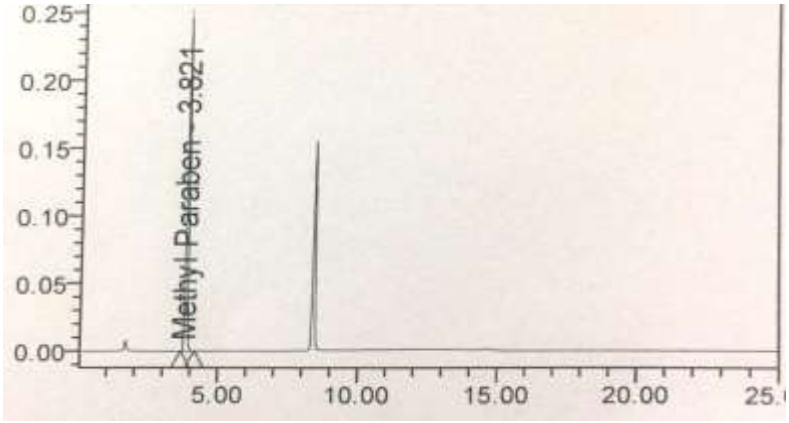


Fig. 2: Chromatogram of methyl paraben

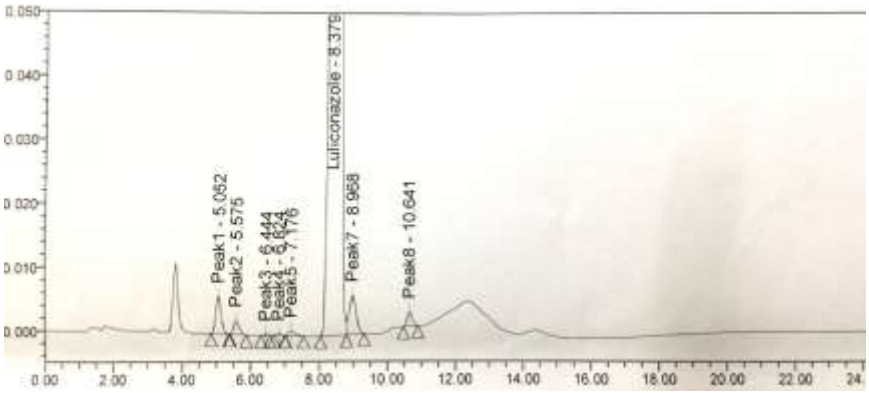
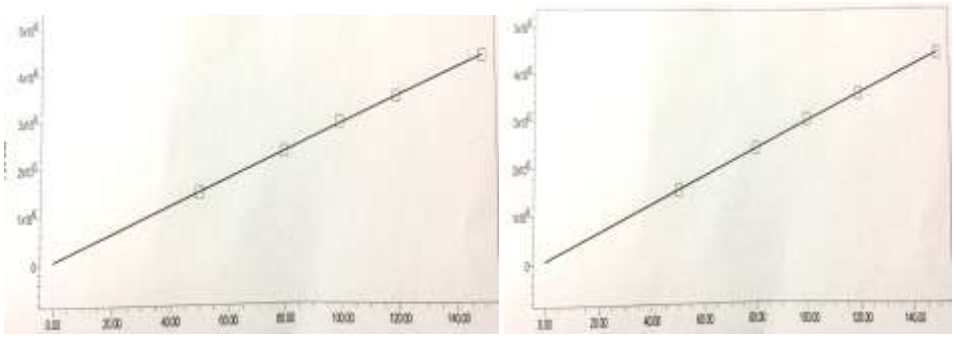


Fig. 3: Chromatogram of luliconazole (Related substances)

Table 6: Related substance for luliconazole

S. No.	Peak name	RT (min)	RRT	Area	% area
1	Peak 1	5.05	0.60	62965	0.20
2	Peak 2	5.57	0.67	23771	0.08
3	Peak 3	6.44	0.77	1028	0.00
4	Peak 4	6.82	0.81	1341	0.00
5	Peak 5	7.18	0.86	9628	0.03
6	Luliconazole	8.38		30721509	99.32
7	Peak 6	8.97	1.07	86121	0.28
8	Peak 7	10.64	1.27	24171	0.08
Avg.				30930535	100.00



(A) Luliconazole (B) Methyl paraben

Fig. 4: Calibration curve of (A) luliconazole and (B) methyl paraben for assay

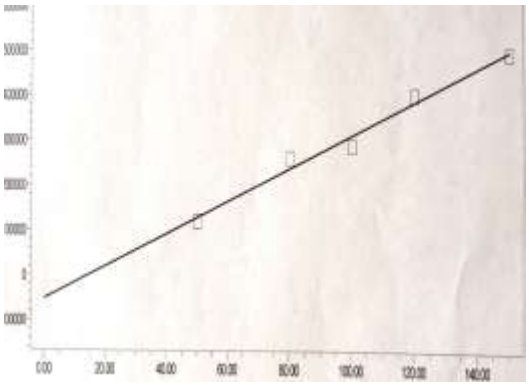


Fig. 5: Calibration curve of luliconazole for related substance

Conclusion

A simple, specific, accurate, precise, rapid, robust and selective stability indicating reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for assay and related substances and validated for quantification of antifungal drug luliconazole with its excipients in its topical dosage form. Forced degradation study is done to determine stability of the product. The mobile phase used ammonium acetate buffer and acetonitrile in (60:40) ratio.

The column used is Inertsil ODS 3 V (150*4.6 mm, 5µm). The retention time for luliconazole and methyl paraben were about 8 min and 4 min. Total impurity of the product was not more than 1.5%. The % assay was 102.7% of luliconazole and 101.3% for methyl paraben and stability study of luliconazole reveals that percentage degradation is within the limit. The product is stable under stress conditions. Hence RP-HPLC method was found to be specific, accurate, robust for simultaneous estimation of luliconazole and methyl paraben. The forced degradation studies states that there is no interference of degraded product. The impurity profiling shows that there was no interference of either of the impurity on main peak .The percentage recovery of luliconazole, methyl paraben and BHT were within limit according to ICH guidelines. The method is precise. The studies showed that the method can be applied in QC laboratory for routine quality check as well as stability studies for formulated product of luliconazole.

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