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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF POSACANAZOLE BY RP-HPLC METHOD

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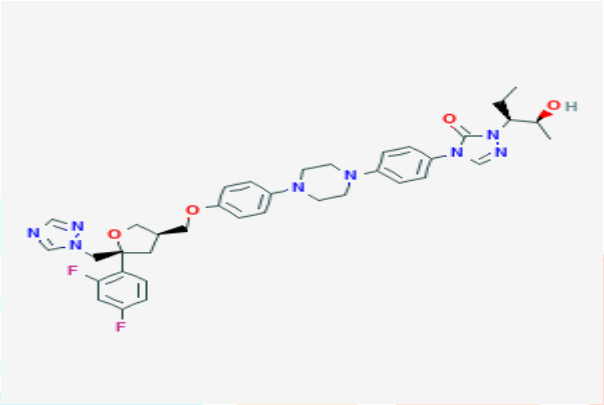
Abstract

A simple, rapid, selective, sensitive, accurate and precise High Performance Liquid Chromatography (HPLC) with UV detection method has been developed and validated for determination of Posaconazole in Human plasma. C18 (250x4.6mm) column was used with the mobile phase containing a mixture of Acetonitrile: Water (55:45v/v). The flow rate was 0.8ml/min and drug was monitored at 262nm. In analytical method development linearity for Posaconazole was found to be 5-100µg/ml with regression coefficient 0.9954. The accuracy and recovery was found within limit. The robustness study was performed and the method was found to be fully robust. Plasma samples were processed using acetonitrile as precipitating agent to extract drug. The linearity for Posaconazole was found to be 0.15 to 50µg/ml with regression coefficient (r2) 0.9955. The recovery was found to be 85.63%. Stability studies for freeze thaw cycle, short term stability and long term stability was done. The % CV for stability study was found within a limit.

Keywords- Posocanazole, Reverse phases HPLC, Accuracy, Precision,

Introduction

Posaconazole is a broad-spectrum, triazole compound, second generation showing antifungal activity. Posaconazole strongly inhibits 14-alpha demethylase, a cytochrome P450-dependent enzyme. Inhibition of 14-alpha-demethylase prevents the conversion of lanosterol to ergosterol, an important component of the fungal cell wall. Inhibition of ergosterol synthesis changes the fungal cell membrane composition and integrity, alters membrane permeability and eventually leads to fungal cell lysis. Compared to other azole antifungals, posaconazole is a significantly more potent inhibitor of sterol 14-alpha demethylase. The aim of this work was to develop a simple, accurate, reproducible and sensitive method for determination of Posaconazole in human plasma using rapid, convenient and simple reverse phase HPLC method.



Materials and methods

Chemicals and reagents

Pure sample of Posacanazole, Acetonitrile (HPLC grade), Water and Drug was obtained from Glenmark Pharmaceuticals.

Instruments used

Sr.No.	Instrument	Make/Model
1	HPLC	Agilent 1120 Compact LC
2	UV Spectroscopy	Shimadzu-1700 UV/VIS
3	Balance	LC/GC
4	Ultrasonic bath	Life care

1. Optimization of Chromatographic conditions

Optimization of the mobile phase was performed based on resolution, asymmetric factor and peak area obtained for Posaconazole. The mobile phase Methanol:water (50:50, 60:40, 70:30) and Acetonitrile(HPLC grade): water (HPLC grade) (30:70, 40:60, 50:50, 55:45,) was also tried. Acetonitrile: Water (55:45 v/v) at a flow rate of 0.8 ml/min was found to be satisfactory and gave symmetric and well resolved peaks for Posaconazole. The chromatogram was recorded at 262.0 nm as spectrum of Posaconazole showed maximum response at this wavelength.Chromatogram showed symmetrical peaks with good shapes; tailing factor for

Posaconazole was within range & the resolution of standard drug was satisfactory. Retention time for Posaconazole was found to be 4.683 min. The system suitability parameters observed by using this mobile phase are reported.

1. **Preparation of mobile phase:**

HPLC grade Acetonitrile:HPLC grade Water (55: 45v/v) which was filtered through 0.45 µm membrane filter and sonicated on ultrasonic bath for 15 min.

. 3. Preparation of standard stock solution:

Posaconazole standard stock solution was prepared by transferring 2.5 mg of Posaconazole working standard into a 25 ml volumetric flask, approximately 10 ml of methanol (HPLC Grade) was added and sonicated for 20 min. the volume was made up to 25 ml with HPLC grade water to get the concentration of 100 µg/ml. This solution was filtered through a 0.45µm pore size nylon 66 membrane.The subsequent dilutions were prepared by diluting stock solution with the methanol.

Separation of plasma from Human Blood:

The blood plasma was collected from Shraddha Laboratory. Blood will be collected into purple top EDTA tubed and centrifuge (3000 rpm) at 4ºC for 20 minutes. After centrifugation use clean pipette technique place 1.0 ml of plasma into 1.5ml Eppendorf tube labeled with tracking number and 'plasma'.

Preparation of sample solution:

Sample solution was prepared by taking 0.90 ml of rat plasma and 100µl of working standard solution of 0.150, 0.5, 1.50, 2.5, 3.5, 5 µg/ml and 1ml of precipitating agent acetonitrile to precipitate plasma protein, were added and mixed. The resulting solution 1.5, 5 , 15, 25, 35, 50 µg/ml was centrifuged at 3000 rpm for 15min. at 2-4ºC.the supernatant layer was separated and analyzed.

Spiking of Posaconazole in plasma

Table 1 . Spiking of Posaconazole in plasma

Concentration (µg/ml)	Vol. of spiking (ml)	Vol. of plasma (ml)	Final vol. (ml)	Final conc. (µg/ml)
5	0.1	0.9	1	0.5
10	0.1	0.9	1	1.0
15	0.1	0.9	1	1.5
25	0.1	0.9	1	2.5
35	0.1	0.9	1	3.5
50	0.1	0.9	1	5.0

Validation Method Development:

The fundamental parameters for Bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability. The measurements for each analyte in the biological matrix should be validated. Typical method development and establishment for Bioanalytical method include determination of

(1) Selectivity,

(2) Accuracy, precision, recovery,

(3) Calibration curve and

(4) Stability of analyte in spiked samples.

1. Selectivity

Analysis of blank sample of the appropriate biological matrix (plasma) should be obtained from at least six sources were tested for interference, & no interference at reported retention time was found.

Calibration curve

The concentration range over, which the linearity was found to be 5-100 µg/ml. The results are shown in Table 2.

Preparation of quality control standards

The quality control standard solution 1.5µg/ml, 25µg/ml, 50µg/ml were prepared.

Accuracy & Precision

Accuracy was measured using three determinations of LQC (1.5µg/ml.), MQC (25 µg/ml.) and HQC (50µg/ml.).

The precision was carried out by within batch intraday& inter batch precision.

Accuracy & Precision within batch

The within batch accuracy & precision was performed in single day by taking three different Concentrations, & each concentration has three determination.

Inter batch Accuracy & Precision

The inter batch accuracy & precision was performed in different days by taking three different concentrations, & each concentration has three determination.

1. Recovery

Recovery experiment should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, high) with unextracted standards that Represent 100% recovery.

2. Stability

a. Freeze & Thaw stability

The freeze–thaw cycle was repeated two more times, and then analyzed on the third cycle.

b. Short term temperature stability

The short term stability was performed by three aliquots of each of the low and high concentrations were tested at room temperature and kept at this temperature from 8 hours and analysed.

Long term stability

Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions for 15 days.

Result and summery:

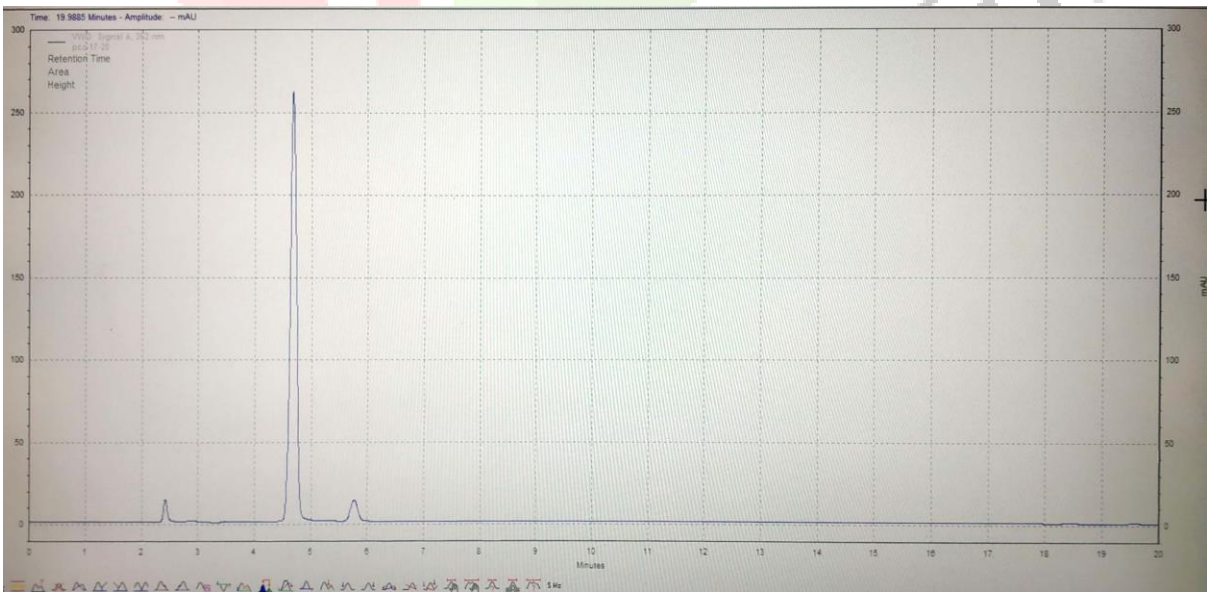


Fig 2: Final chromatogram of posaconazole

Table 2: Linearity of Posaconazole.

Standard calibration data of posaconazole

Sr. No	Concentration (µg/ml)	Peak Area* (mAU)	S.D
1	0.5	296797	0.215
2	1.0	724593	0.0325
3	1.5	1129352	0.0227
4	2.5	1603231	0.0743
5	3.5	1992591	0.0518
6	5.0	2511290	0.0725

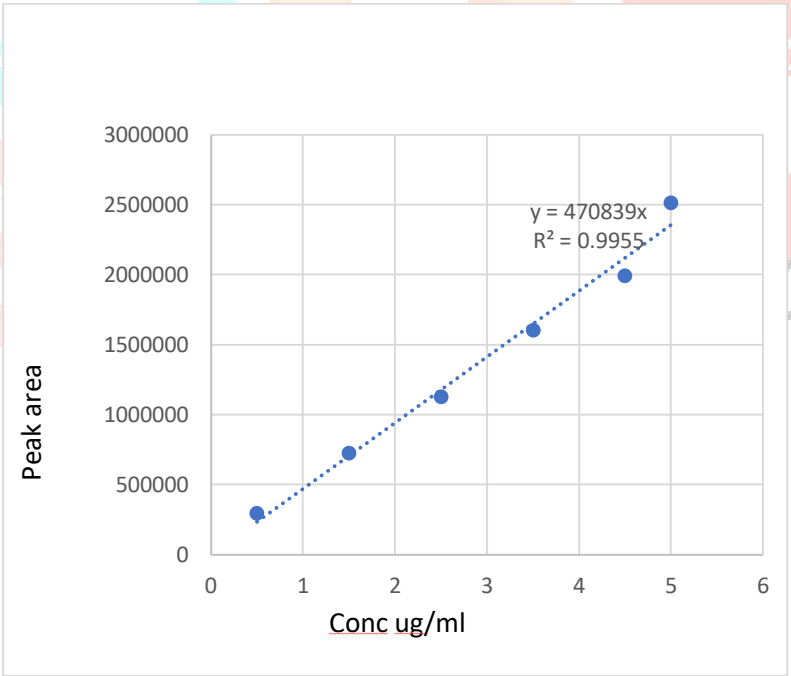


Fig 1: Calibration curve of Posaconazole spiked in plasma

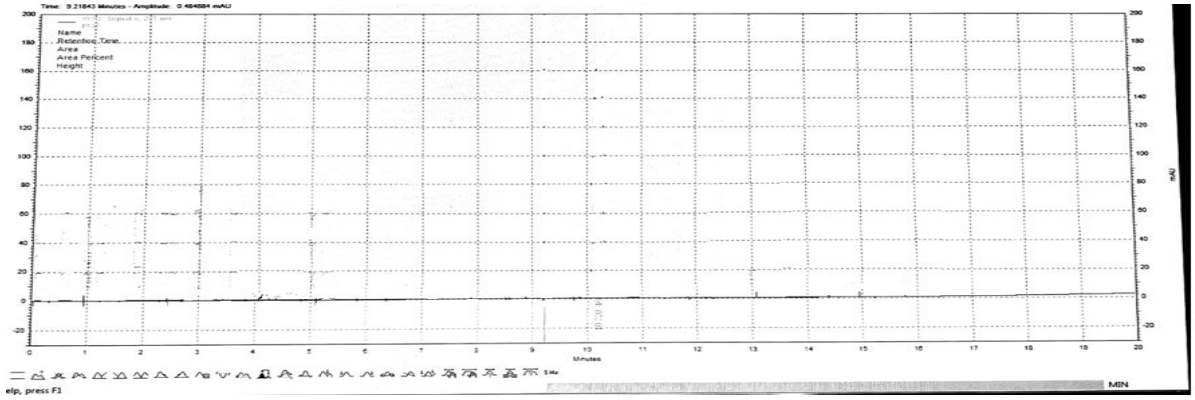


Fig 3: Chromatogram of plasma.

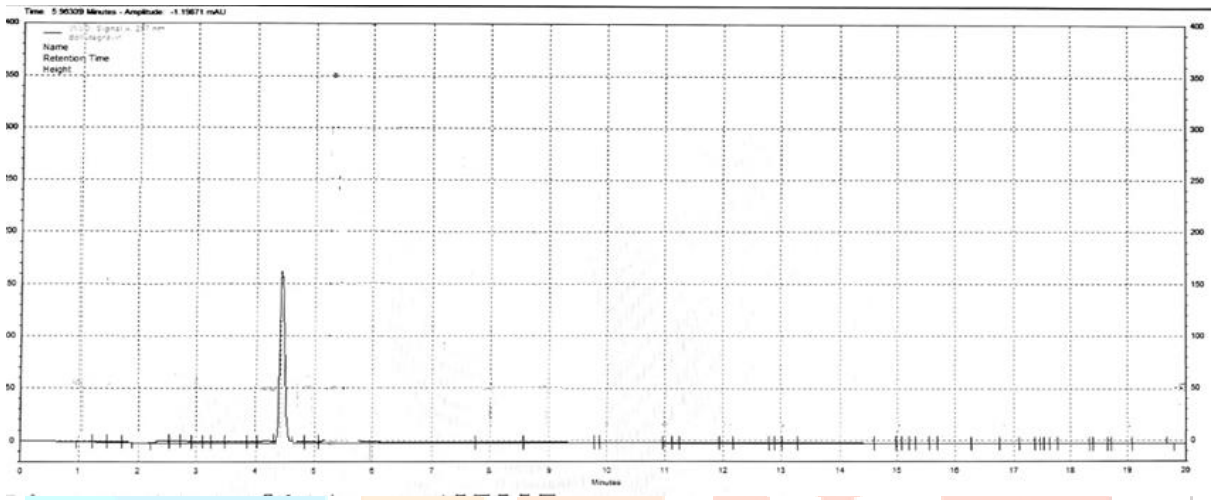


Fig 4 : Chromatogram of LQC Sample spiked in plasma.

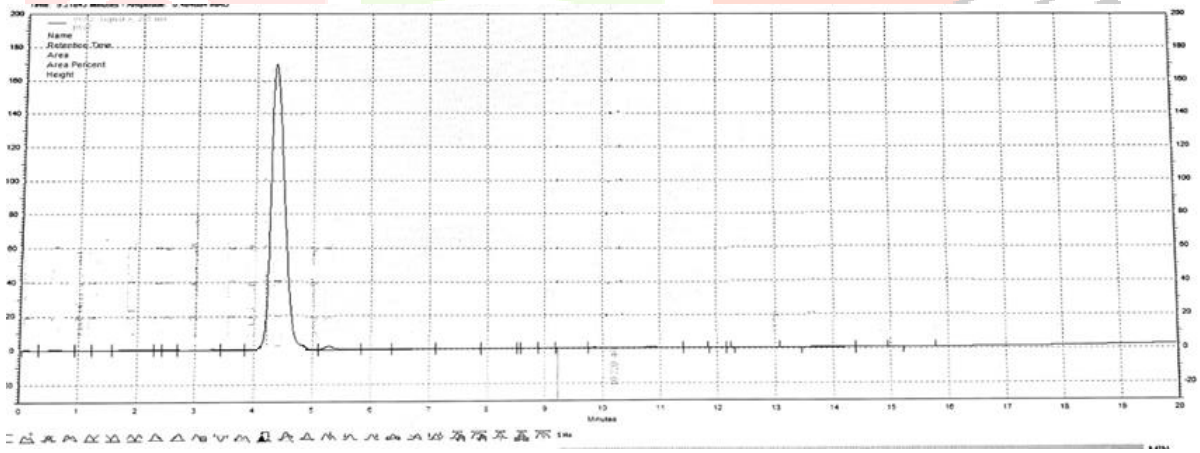


Fig 5 : Chromatogram of MQC sample spiked in plasma.

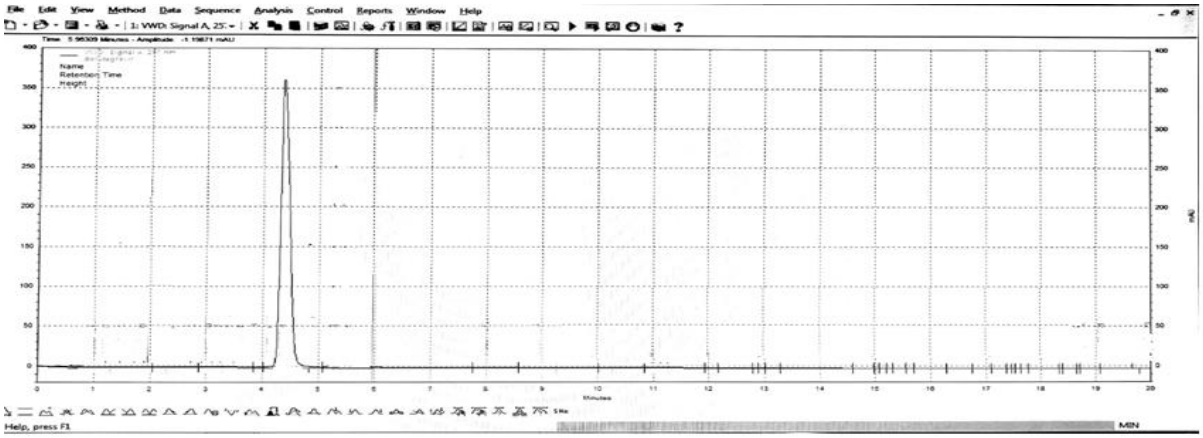


Fig 6 : Chromatogram of HQC sample spiked in plasma.

Table 3: Accuracy and Precision within batch

Quality control sample	Amt. Added (µg/ml)	Peak Area*	Amt found (µg/ml)	% Accuracy	%C.V
LQC	0.5	296796	0.49	98.15	0.2280
	0.5	296489	0.48	97.12	
	0.5	296199	0.49	98.15	
MQC	2.5	1129352	2.46	98.43	0.4407
	2.5	1139126	2.47	98.88	
	2.5	1139489	2.48	98.82	
HQC	5	2511290	4.96	99.92	0.6179
	5	2512671	4.89	98.47	
	5	2509612	4.87	97.94	

***Average of three determination**

Table 4: Inter batch Accuracy and Precision

Quality control sample	Amt. Added (µg/ml)	Peak Area*	Amt. found (µg/ml)	% Accuracy	% C. V
LQC	0.5	269796	0.48	98.00	0.2391
	0.5	296482	0.47	98.45	
	0.5	296191	0.49	96.90	
MQC	2.5	1129352	2.49	96.56	0.2449
	2.5	1139484	2.46	96.98	
	2.5	1139216	2.47	98.80	
HQC	5	2511296	4.89	98.98	0.6052
	5	2512671	4.87	98.81	
	5	2509612	4.86	98.44	

***Average of three determinations**

Table 5: Recovery study

Conc. (µg/ml)	Peak Area* (Extracted)	Peak Area* (Un-extracted)	% Recovery
0.5	296796	339896	87.31%
2.5	1129352	1318789	85.63%
5	2511290	2945278	85.26%

***Average of three determinations**

Stability:

Table 6 : Freeze and Thaw stability

Conc. (µg/ml)	Peak Area	Conc. Found	% Purity*	S.D	% C.V
0.5	296421	0.48	98.72	0.3295	0.2430
2.5	1129322	2.49	99.33	0.2468	0.4486

***Average of three determinations**

Short term stability: -

Table 7: Short term temperature stability

Conc. (µg/ml)	Peak Area	Conc. Found (µg/ml)	% Purity	S.D	% C.V
0.5	296221	0.48	98.70	0.2100	0.2114
2.5	1129355	2.48	99.12	0.4661	0.4761

Table No. 7.32 Long Term Stability

Conc. (µg/ml)	Peak Area	Conc. Found (µg/ml)	% Purity	S.D	% C.V
0.5	296421	0.49	99.1	0.31121	0.2225
2.5	1129322	2.47	98.82	0.2900	0.4982

Conclusion

Bioanalytical method for Posaconazole has been developed and method was validated as per USFDA guideline. The proposed methods were found to be simple, accurate, precise and reproducible and can be applied for analysis of drug in rat plasma. The proposed method was also applied for the estimation of bioavailability, bioequivalence, pharmacokinetic & toxicokinetic data of Tablet formulation.

Acknowledgement

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