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Method Development and Validation for the Determination of Related Compounds in Posaconazole by RP-HPLC

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ABSTRACT

An accurate and precise HPLC method was developed for the determination of related compounds in Posaconazole. Separation of the drug was achieved on Inersil ODS-2, 250 x 4.6 mm, 5.0 µm or equivalent column using a mobile phase consisting of Phosphate buffer (pH 62.5): Acetonitrile(20:80). The flow rate was 0.8 mL/minute and the detection wavelength was 260 nm..The proposed method was validated for its linearity, accuracy, precision and robustness. This method can be employed for routine quality control analysis of TDA, PDC, TMB in Posaconazole.

KEY WORDS : Posaconazole TDA, PDC, TMB, Stability, Method development, RP-HPLC, Validation

Introduction:

Posaconazole, sold under the brand name Noxafil among others, is a triazole antifungal medication. It was approved for medical use in the United States in September 2006, and is available as a generic medication. Posaconazole is used to treat invasive *Aspergillus* and *Candida* infections.[5] It is also used for the treatment of oropharyngeal candidiasis (OPC), including OPC refractory to itraconazole and/or fluconazole therapy. It is also used to treat invasive infections by *Candida*, *Mucor*, and *Aspergillus* species in severely immunocompromised patients.Clinical evidence for its utility in treatment of invasive disease caused by *Fusarium* species (fusariosis) is limited.It appears to be helpful in a mouse model of naegleriasis. Posaconazole works by disrupting the close packing of acyl chains of phospholipids, impairing the functions of certain membrane-bound enzyme systems such as ATPase and enzymes of the electron transport system, thus inhibiting growth of the fungi. It does this by blocking the synthesis of ergosterol by inhibiting of the enzyme lanosterol 14α-demethylase and accumulation of methylated sterol precursors. Posaconazole is significantly more potent at inhibiting 14-alpha demethylase than itraconazole.

Chemically it is 4-(4-(4-(4-(((3*R*,5*R*)-5-(2,4-difluorophenyl)-5-(1,2,4-triazol-1-ylmethyl)oxolan-3-yl)methoxy)phenyl)piperazin-1-yl)phenyl)-2-((2*S*,3*S*)-2-hydroxypentan-3-yl)-1,2,4-triazol-3-one. It is a white powder and soluble in Acetonitrile, Methanol. Insoluble in Water.

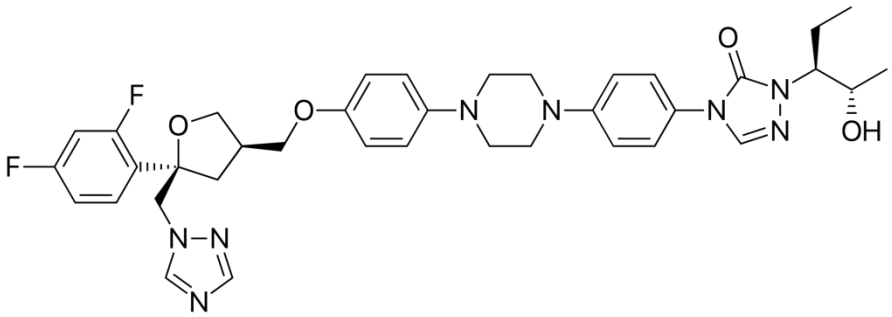

Fig.1: Chemical structure of posaconazole

Some of the active pharmaceutical ingredients may contain impurities which are not known to us. It is necessary to identify such impurities as they may effect our health or they may decrease the pharmacological activity of the main drug. So, we have to develop method for the related impurities of our product. The need to develop new analytical methods for assurance of quality, safety and efficacy of drugs and pharmaceuticals is quite important because of their use not only as health care products but also life saving substances. The analytical methods assume of great importance due to development of new drugs, continuous changes in manufacturing processes for existing drugs and setting up of threshold limits for individual and total impurities of drugs by regulatory authorities. Keeping this in view, an attempt was made in the present investigation to develop new RP-HPLC method for the estimation of Posaconazole and its related impurities(TDA, PDC, TMB). PCL is a triazole which performs Antifungal activity respectively. The newly developed

HPLC method would be then validated as per ICH Guidelines to indicate that the analytical procedure employed is recommended to be suitable for its routine analysis in terms of various parameters like specificity, LOD, LOQ, linearity, precision, accuracy, system suitability.

Materials and Methods:

Equipment

The Method development and Validation was carried out using Waters Alliance-HPLC system equipped with waters 1525 binary HPLC pump, 2695-separation module connected to 2996-photo diode array detector, and Waters 2707 auto sampler. The data was acquired by Empower® version 2. The other equipment used were Ascoset Electronic balance, ADWA pH meter, heating mantle. Ultrasonic bath was used for sonication of the samples. Hot air oven was used to carry out thermal degradation studies. UV cross linker, with series of 23400 model UV chamber, equipped with a UV fluorescence lamp with the wavelength range between 200 & 300 nm was used for photo degradation studies.

Chemicals and Reagents

Posaconazole working standard was kindly given as gift sample by Mylan labs Pvt. Ltd, Hyderabad. HPLC grade solvents include acetonitrile, water and methanol. Analytical grade chemicals include sodium hydroxide, hydrochloric acid, 20% hydrogen peroxide, Ortho phosphoric acid, Triethyl amine and potassium dihydrogen phosphate were purchased from E. Merck Limited, Mumbai, India.

Chromatographic conditions

HPLC analysis was carried out on Waters Alliance-HPLC system equipped with 2695-separation module connected to 2996-photo diode array detector and the data was acquired by Empower® version 2. Separation was achieved using Inersil ODS-2, 250 x 4.6 mm, 5.0 µm as a column with mobile phase of pH 3.0 phosphate buffer and Acetonitrile in gradient mode of elution. The samples were analyzed using 10 µL injection volume, Flow rate was maintained at 0.8 mL/min with runtime of 60 min and the temperature was maintained at 30°C throughout the analysis. Detection and purity establishment of the drugs were achieved using PDA detector at 260 nm wavelength.

Table : 1 Gradient programme:

Time(min)	Solvent-A (%)	Solvent-B (%)
0.01	80	20
5.00	80	20
35.0	50	50
45.0	10	90
50.0	10	90
55.0	80	20
60.0	80	20

Preparation of standard solution:

Weigh accurately about 20.0 mg of PCL reference standard into a 20 mL volumetric flask, add 1.0 mL of reference stock solution dissolve and dilute to the volume with diluent and mix.

Preparation of impurities standard solutions:

Weigh accurately each 3.0 mg of TDA, PDC, TMB reference standards into a 100 mL volumetric flask, dissolve and dilute to the volume with diluent and mix. Take 1.0 mL reference stock solution into a 20 mL volumetric flask, dissolve and dilute to the volume with diluent and mix.

Method Validation

The developed and optimized RP-HPLC method was validated according to international conference on harmonization (ICH) guidelines Q2(R1) in order to determine the system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, ruggedness and robustness.

System suitability

System suitability parameters were evaluated to verify system performance. 20 µL of standard solution of posaconazole and its impurities was injected five times into the chromatograph, and the chromatograms were recorded. Parameters such as number of theoretical plates and peak tailing were determined.

Specificity

The specificity of the analytical method was established by injecting the solutions of diluent (blank), placebo, working standards and sample solutions of posaconazole and its impurities individually to investigate interference from the representative peaks.

Precision

Repeatability/ method precision was performed by injecting six replicates of same concentrations of posaconazole and its impurities, calculated % assay and %RSD. Reproducibility/ Ruggedness/ Intermediate precision was performed using different analysts and a different instrument in the same laboratory.

Accuracy

Accuracy of the proposed method was determined using recovery studies by spiking method. The recovery studies were carried out by adding known amounts (80%, 100% and 120%) of the working standard solutions of posaconazole and its impurities to the pre-analysed sample. The solutions were prepared in triplicates to determine the accuracy.

Linearity

Linearity was evaluated by analyzing different concentrations of the standard solutions of posaconazole and its impurities. Five working standard solutions ranging between 12.5µg/mL-100µg/mL were prepared and injected. The response was a linear function of concentration over peak area and were subjected to linear least-squares regression analysis to calculate the calibration equation and correlation coefficient.

Limit of detection and Limit of quantification: Limit of detection (LoD) and limit of quantification (LoQ) of posaconazole and its impurities were determined by calibration curve method. Solutions of posaconazole and its impurities were prepared in linearity range and injected (n = 3).

Robustness

To examine the robustness of the developed method, experimental conditions were deliberately changed, resolution, tailing factor, and theoretical plates of posaconazole and its impurities peaks were evaluated. To study the outcome of the flow rate on the developed method, it was changed ± 0.2mL/minute. The effect of column temperature on the developed method was studied at ± 5°C, organic phase composition in mobile phase was changed ±10% and pH of the buffer is changed ±0.2. In all the above varied conditions, the composition of aqueous component of the mobile phase was held constant.

Results and Discussion

Optimized method:

All the Posaconazole possible process impurities are well separated in this method with good resolution. The resolution between PDC and TMB is more than **2.0.** The USP tailing factor for all peaks is less than 2.0. The detection wave length was optimized by using the Photo Diode Array detector (PDA) and it is found that Posaconazole and related impurities have maximum response at 260 nm wave length.

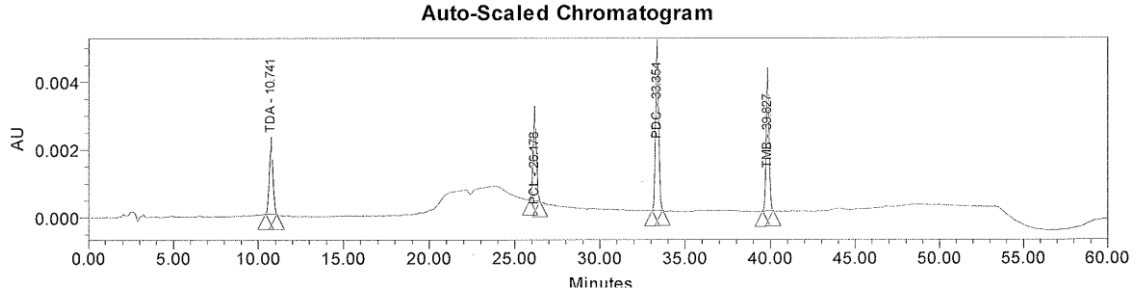

Fig. 2: Chromatogram of standards

Table : 2 The relative retention times of impurities w.r.t to Posaconazole peak:

S.No	Name of the compound	~ RT (Minutes)	~ RRT
1	TDA	10.74	0.41
2	PCL	26.18	1.00
3	PDC	33.35	1.27
4	TMB	39.83	1.52

Assay:

Amount Present $= \frac{At}{As} \times \frac{Ws}{Ds} \times \frac{Dt}{Wt} \times$ **Avg.Wt** X $\frac{PA}{100}$

Assay $= \frac{\text{Amount obtained}}{\text{Label Claim}} \times 100$

At = Area of sample, As = Area of standard

Ws = Weight of standard, Wt = Weight of sample (1.013gms)

Dt = Dilution of sample, Ds = Dilution of standard

PA = Potency of standard Posaconazole 99.9%

Table: 3. Results of %Assay of TDA, PDC, TMB:

Name	As	At	Wt. equivalent taken (mg)	%Assay
TDA	30552	21531	5.0	0.09
PDC	62861	25124	5.0	0.07
TMB	48545	28715	5.0	0.07

%Assay ofTDA, PDC, TMB in Posaconazole was found to be 0.09, 0.07, 0.07% and which are within acceptance criteria 95-105%

System Suitability

Table 4: System suitability results for posaconazole and its impurities

S.No	Name	Retention time	Area	USP Tailing	USP Plate Count
1	TDA	10.74	30552	1.0	8900
2	PCL	26178	26178	1.02	6750
3	PDC	33.354	62861	1.06	11562
4	TMB	39.827	48545	1.0	23300

% RSD should not be more than 2.0 % for area .Tailing factor should not be more than 2 %. Plate count should not be less than 3000. It was observed from the data tabulated above that all the system suitability parameters meet the predetermined acceptance criteria as per the test method and indicates the suitability of the selected system.

Linearity:

Table : 5 Linearity for TDA

Level	Concentration in % (X axis)	Area
1	0.0002	2985
2	0.0008	17285
3	0.0011	25772
4	0.0015	34342
5	0.0019	45046
6	0.0023	52339
Correlation coefficient		**0.9994**
Intercept		**-661**
% Y Intercept		**-1.26**

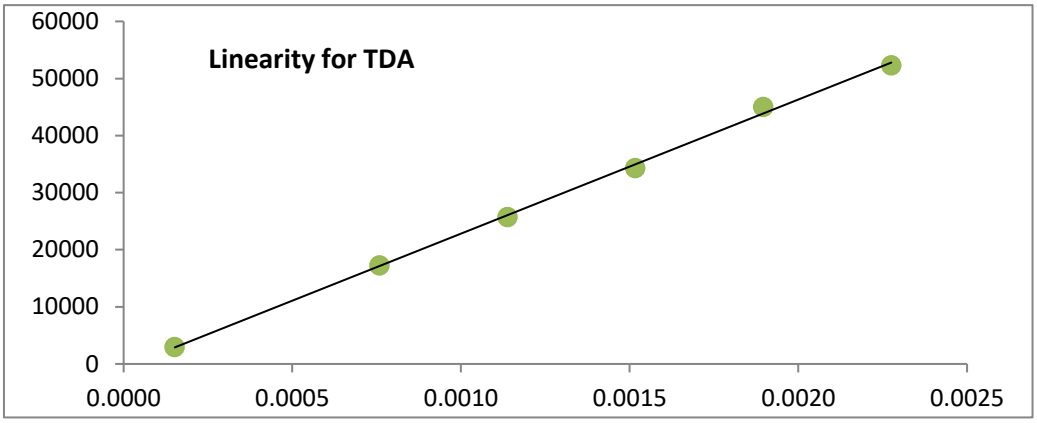

Figure 3 Linearity for TDA

Table : 6 Linearity for PCL:

Level	**Concentration in % (X axis)**	**Area**
1	0.0001	2662
2	0.0005	17432
3	0.0008	26009
4	0.0010	37108
5	0.0013	47146
6	0.0015	56260
Correlation coefficient		**0.9991**
Intercept		**-1269**
% Y Intercept		**-2.26**

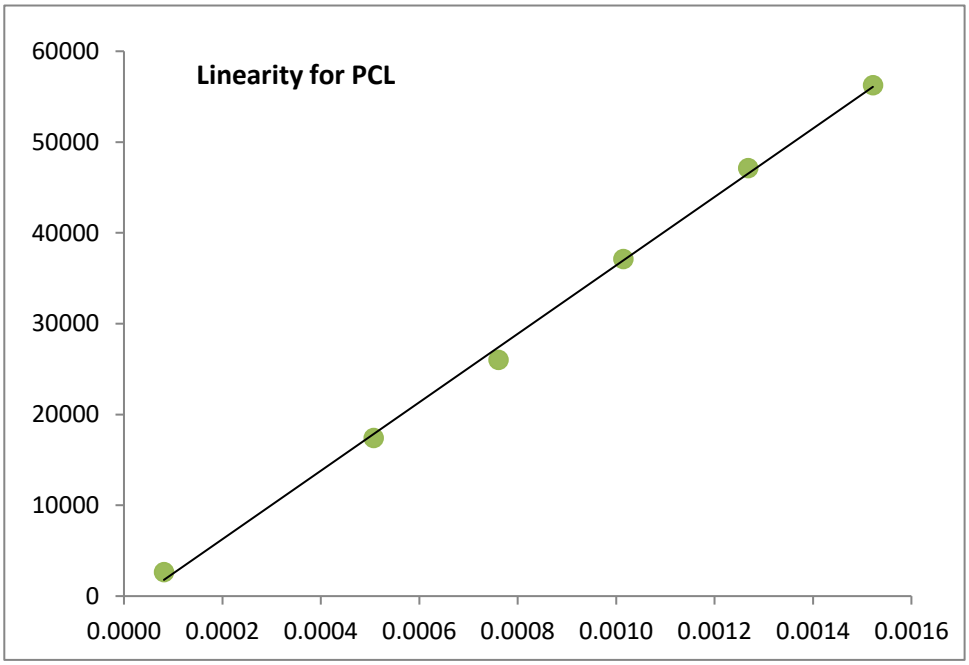

Figure : 4 Linearity for PCL

Table : 7 Linearity for PDC:

Level	Concentration in % (X axis)	Area
1	0.0001	2777
2	0.0008	31112
3	0.0011	44066
4	0.0015	66145
5	0.0019	77085
6	0.0023	91337
Correlation coefficient		**0.9975**
Intercept		**6**
% Y Intercept		**0.01**

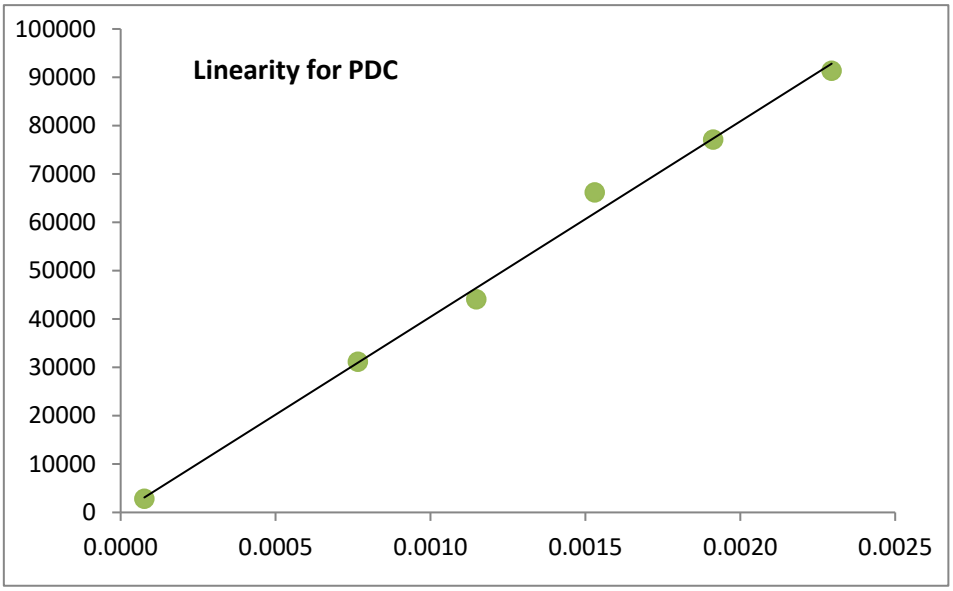

Figure : 5 Linearity for PDC

Table : 8 Linearity for TMB:

Level	Concentration in % (X axis)	Area
1	0.0001	1939
2	0.0008	25057
3	0.0011	36553
4	0.0015	51408
5	0.0019	63309
6	0.0023	75482
Correlation coefficient		**0.9995**
Intercept		**-525**
% Y Intercept		**-0.70**

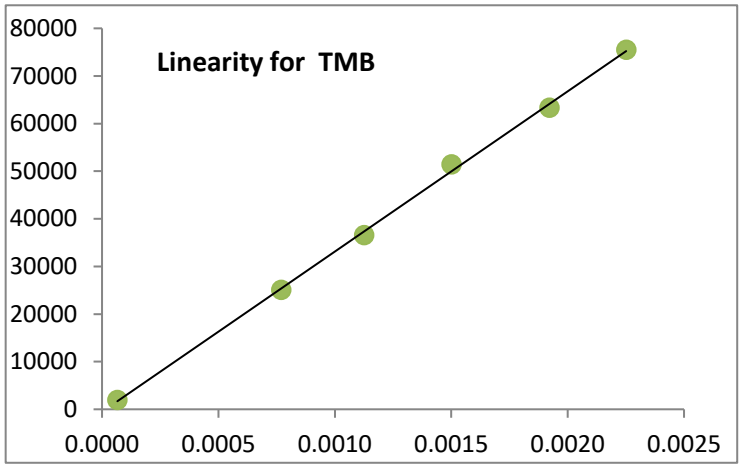

Figure : 6 Linearity for TMB

The correlation coefficient should not be less than 0.999**.** Correlation coefficient value for each component was within limit. (More than 0.99).

Precision

Table 9 : Precision

S.NO	Injection Number	Peak area for Posaconazole	Acceptance Criteria
1	Standard 1	2440913	The %RSD for Posaconazole peak area of from six replicate injections of standard solution should not be more than 2.0
2	Standard 2	2409496	
3	Standard 3	2404314	
4	Standard 4	2407286	
5	Standard 5	2412208	
6	Standard 6	2421153	
Mean		2415895	
%RSD		0.9	

The Relative standard deviation of individual area of Posaconazole from six standard preparations should be not more than 2.0%. The Relative standard deviation of individual area of Posaconazole was found to be 0.9 respectively. Results were found to be within limits.

Specificity

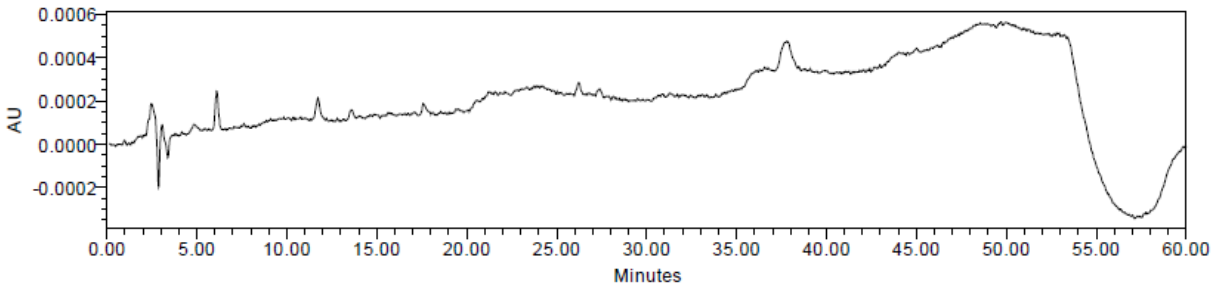

Figure : 7 Blank Chromatogram:

Each known impurity solution and Posaconazole standard solution was prepared individually at target concentration of the test sample. A solution of all known impurities spiked with the Posaconazole test sample (Blend solution) was also prepared. All these solutions were analyzed using the PDA detector as per the HPLC method.

The specificity of the method was evaluated by injecting blank, Standard Solution and the sample solution prepared as per the proposed method to check for interference, if any, at the retention time of posaconazole and impurities peak from any peak due to blank. It was found that there was no interference of blank at the posaconazole and impurities peak RT.

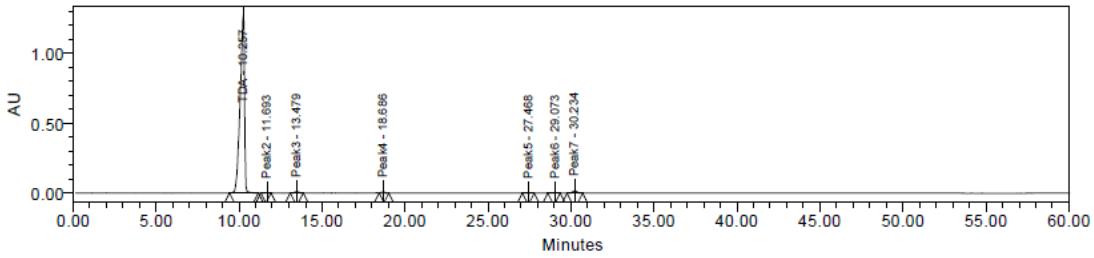

Figure :8 TDA solution chromatogram:

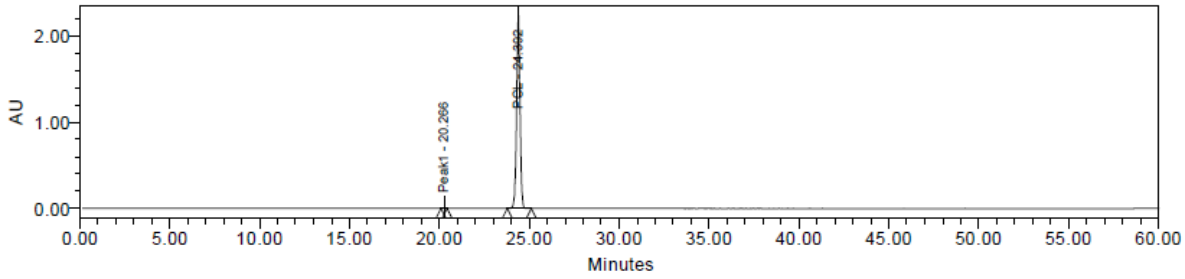

Figure : 9 PCL solution chromatogram:

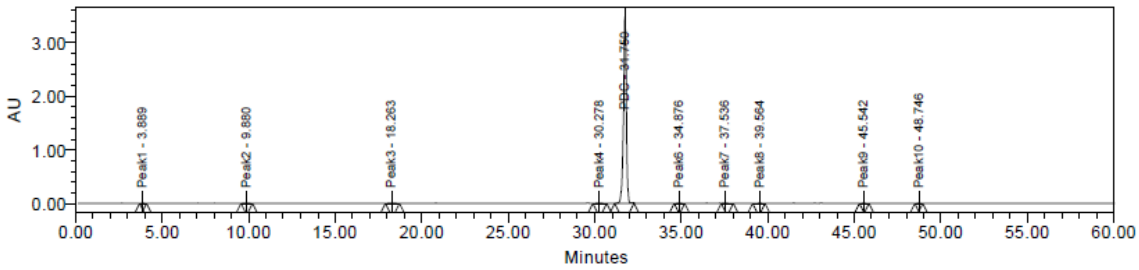

Figure : 10 PDC solution chromatogram:

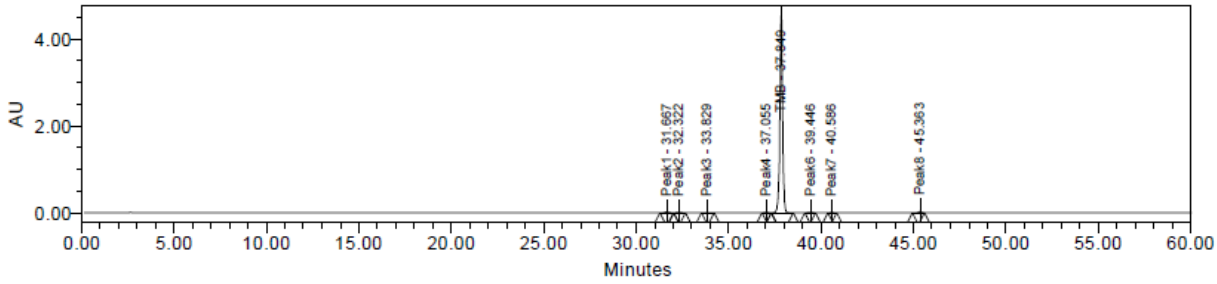

Figure : 11 TMB solution chromatogram:

No interference was observed due to the blank at the retention time of Posaconazole and it's known impurities peaks. Peak purity passed for all impurities obtained from individual solutions.

It is observed that the proposed method is specific and capable to separate all the impurities.

Table : 10 Retention time of individual solution

Component	**Retention time of individual solution**
TDA	10.26
PCL	24.40
PDC	31.75
TMB	37.85

Robustness:

Robustness of the proposed analytical method was evaluated by making deliberate changes in the chromatographic system method parameters, the standard solution and test solutions were injected for each of the changes made to access the robustness of proposed analytical method. Change in column temperature, change in flow rate and change in column. All the system suitability parameters were well within the acceptance criteria. Hence, it was concluded that the method is robust**.**

Table :11 pH Variation Results:

Name of the Impurity	**Initial pH 3.0 results (%)**	**pH 2.8 results (%)**	**Variation**	**pH 3.2 Content (%)**	**Variation**
TDA	0.09	0.11	0.02	0.10	0.01
PDC	0.17	0.14	0.03	0.14	0.03
TMB	0.14	0.13	0.01	0.14	0.00
MSUI	0.12	0.12	0.00	0.11	0.01
TI	0.63	0.66	0.03	0.61	0.02

Table : 12 Temperature Variation Results:

Name of the Impurity	**Initial 35 ºC results (%)**	**33 ºC results (%)**	**Variation**	**37 ºC results (%)**	**Variation**
TDA	0.09	0.10	0.01	0.10	0.01
PDC	0.17	0.18	0.01	0.18	0.01
TMB	0.14	0.14	0.00	0.15	0.01
MSUI	0.12	0.14	0.02	0.15	0.03
TI	0.63	0.66	0.03	0.69	0.06

Ruggedness (Intermediate precision)

Established the precision study on a different day with different instrument with freshly prepared solutions. The results obtained from method precision study and Intermediate precision were comparable.

Table : 13 Ruggedness Results:

Name of the Impurity	**Initial results (%)**	**Method Precision results (%)**	**Variation**
TDA	0.09	0.10	0.01
PDC	0.17	0.13	0.04
TMB	0.14	0.12	0.02
MSUI	0.12	0.12	0.00
TI	0.63	0.47	0.16

Limit of Detection and Limit of Quantitation:

The results obtained from method precision study and Intermediate precision were comparable.

Table : 14 Results of LOD and LOQ

S.No.	NAME	LOD(µg/ml)	LOQ(µg/ml)
1	TDA	5	14.9
2	PDC	3.1	10.5
3	TMB	4.2	13.8

Accuracy:

The mean % Recovery of TDA, PDC and TMB at each level should be not less than 95.0% and not more than 105.0%. The %RSD of recovery of TDA, PDC and TMB from the three sample preparations at 50% and 150% levels should not be more than 5.0%.

ACCURACY:

Table 15: Accuracy for TDA, PDC and TMB

% Spiked	Weight added (mg)			Weight Recoverd(mg)			% Recovery		
	TDA	PDC	TMB	TDA	PDC	TMB	TDA	PDC	TMB
50	2.50 2.50 2.52	6.26 6.38 6.23	3.50 3.50 3.52	2.46 2.48 2.49	6.33 6.29 6.46	3.46 3.48 3.49	98.40 99.20 98.81	101.12 98.59 103.69	99.40 100.20 99.81
100	5.08 5.12 5.06	12.54 12.56 12.58	6.08 6.12 6.06	4.88 4.98 4.76	12.47 12.79 12.29	5.88 5.98 5.76	96.06 97.26 97.07	99.44 101.83 97.69	97.06 98.26 98.07
150	7.57 7.73 7.58	18.89 18.73 18.71	8.57 8.73 8.58	7.39 7.68 7.47	18.79 18.83 18.66	8.39 8.68 8.47	97.62 99.35 98.55	99.47 100.53 99.73	98.62 100.35 99.55

Forced Degradation study for Posaconazole:

Posaconazole sample was forcibly degraded under the stress conditions mentioned in the below table .The details of degradation were recorded. The mother sample and forcibly degraded samples of Posaconazole was analyzed for Description, Related substances by HPLC as per the final method. The results of the analysis are as follows:

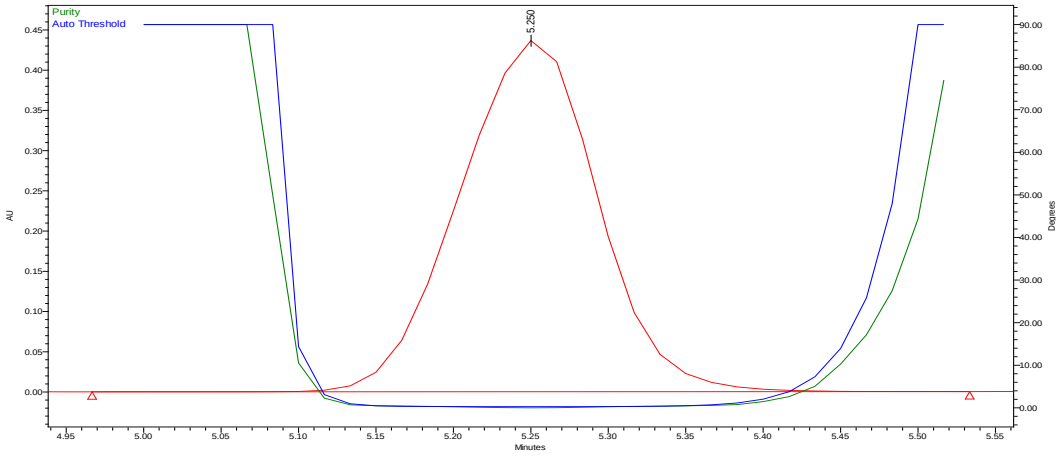

Figure : 12 A. Purity plot of Posaconazole

Thermal Degradation:

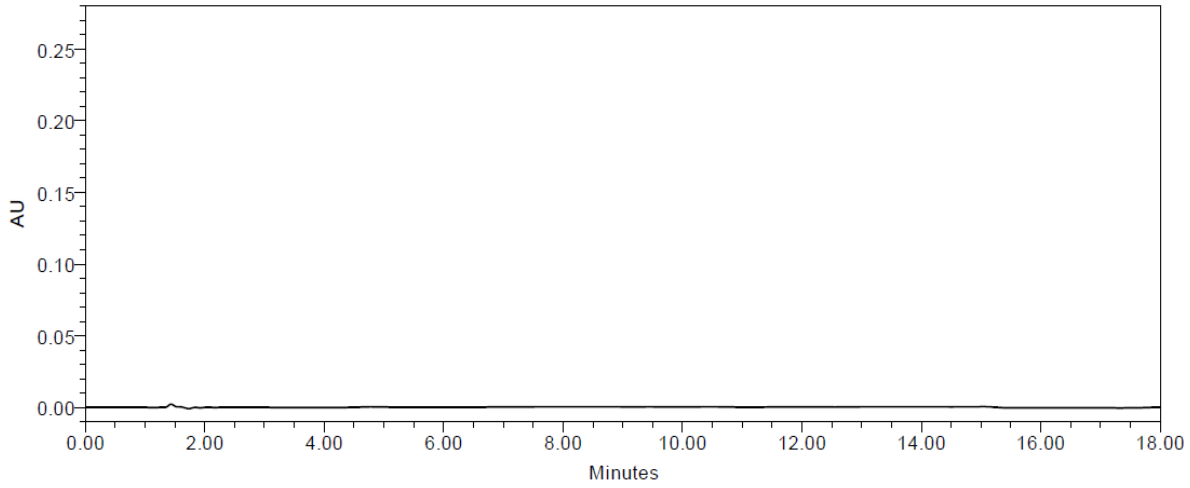

Figure : 13 Typical chromatogram of placebo after Thermal degradation

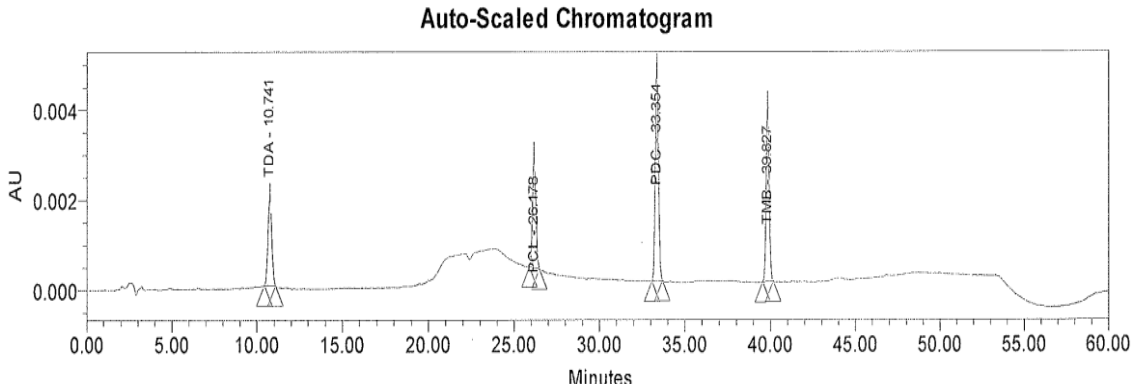

Figure : 14 Purity plot of Posaconazole

Chemical degradation:

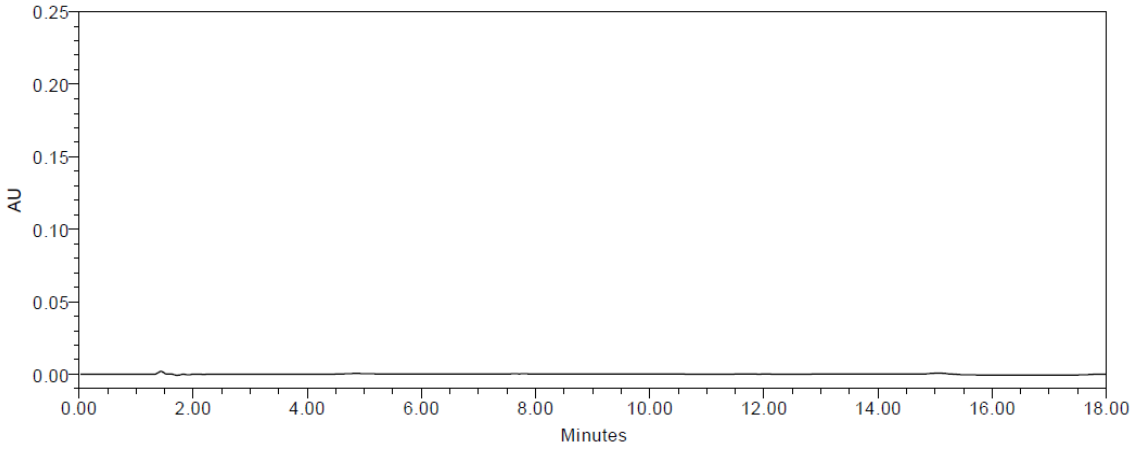

Figure : 15 Acid degradation

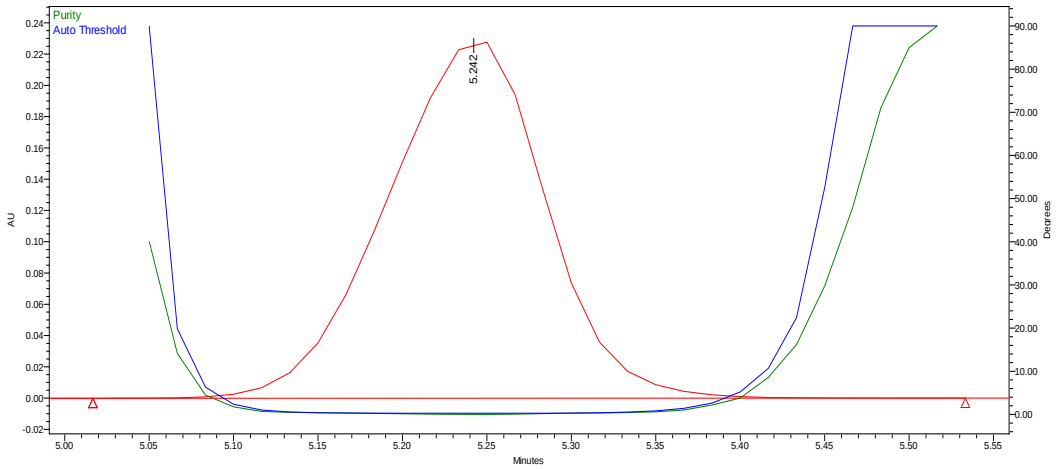

Figure : 16 Peak purity plot for Posaconazole in Acid stressed sample

Table : 16 Physical appearance of samples

Name of the sample	**Period of exposure**	**Physical appearance**
Mother sample (as such sample)	--	White powder
Thermal sample	2 Hours	White powder
UV exposed sample	2 Hours	White powder
Solution in 1.0 N HCl at 80° C	2 Hours	Clear , No change
Solution in 1.0 N NaOH at 80° C	2 Hours	Clear , No change
Solution in Water at 80°C	2 Hours	Clear , No change
Solution in 10 % w/w peroxide at 80° C	2 Hours	Clear , No change
Sunlight exposed solution	2 Hours	Clear , No change

Observation & Inference:

No change is observed in the description of the samples in any of the conditions.

Posaconazole solid state and solution stress conditions and results:

Solid state and solution samples of Posaconazole were prepared and stressed under the conditions described in the above table. The samples were analyzed by HPLC using the preliminary HPLC conditions. The solid state and solutions were prepared approximately 1.0 mg/mL concentration level, All above samples are analyzed in PDA detector and evaluated peak purity for Posaconazole peak. The results are as follows

Table : 17 Degradation conditions and Results (Solid State):

Name of the Sample	Mother sample (As such)	Thermal sample (at 80 °C)	UV light exposure sample
% of TDA	ND	ND	ND
% of PDC	ND	ND	ND
% of TMB	ND	ND	ND
% of MSUI	0.11	0.09	0.11
% of TI	0.11	0.12	0.12
Peak purity	Pass	Pass	Pass

Conclusion:

In the present work, an attempt was made to provide a newer, sensitive, simple, accurate and low cost RP-HPLC method. It is successfully applied for the determination of Related compounds of Posaconazole without the interferences of other constituent in the formulations.

In HPLC method, HPLC conditions were optimized to obtain, an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to get good optimum results. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, capacity factor), run time etc. The system with Water: Acetonitrile(10:90) with 0.8 ml/min flow rate is quite robust.

The developed HPLC method is having the following advantages:

The standard and sample preparation requires less time. No tedious extraction procedure was involved in the analysis of formulation. Suitable for the analysis of raw materials, applicable to dissolution studies and can be used for the content uniformity studies.

Hence, the chromatographic method developed for Related compounds of Posaconazole is said to be rapid, simple, specific, sensitive, precise, accurate and reliable that can be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, bio-pharmaceutics and bio-equivalence studies and in clinical pharmacokinetic studies.