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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF POSACONAZOLE AND ITS IMPUTRIES IN API, DOSAGE FORM BY USING 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.

KEYWORDS: Posaconazole, Phosphate Buffer, Acetonitrile, Intersil, API.

INTRODUCTION
Posaconazole is a recently marketed triazole. The common mode of action of azoles is the inhibition of 14a-demethylase (CYP51), an enzyme important in the ergosterol biosynthesis of yeasts and moulds. Posaconazole is a broad-spectrum azole, including Aspergillus and Candida species, as well as many fun-gi that are resistant to most other antifungal agents, and can be used as an alternative in salvage therapy.[1-3] Posaconazole is well tolerated after oral appli-cation in daily doses of 600–800 mg, whereas doses beyond 800 mg did not result in an increase of the area under the curve (AUC). AUC can be augmented when it is administrated with fatty meals by 400% [4-6]. Although being an inhibitor of CYP3A4, posa-conazole is metabolized by glucuronisation. There-fore, its potential for drug interactions is low, it has a considerably improved and smaller drug interaction profile compared with other triazoles, such as vori-conazole.[7] Due to variable systemic avail-ability known from the chemically related itraconazole, it could be important to measure blood levels, especially in patients with poor absorption, such as cancer patients or patients undergoing abdominal surgery. Because posaconazole is only available for oral application, intestinal resorption may be influenced by extensive abdominal surgical procedures or mucositis.

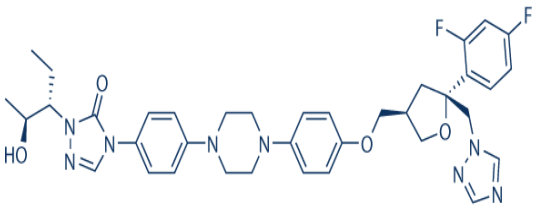

Fig. 1: Chemical structures of Posaconazole.

Impurities

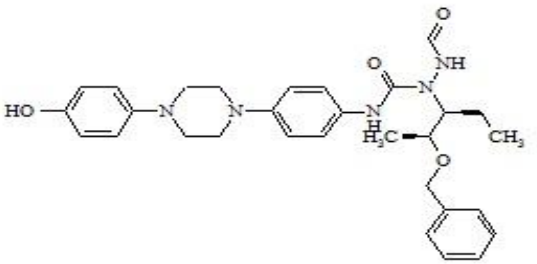

Fig. 2: Chemical structure of TDA.

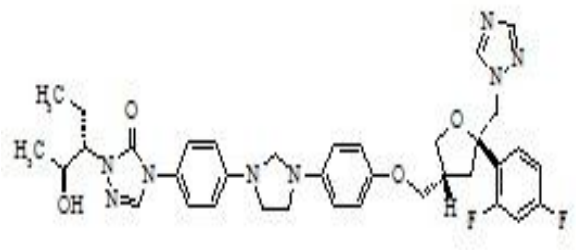

Fig. 3: Chemical structure of PDC.

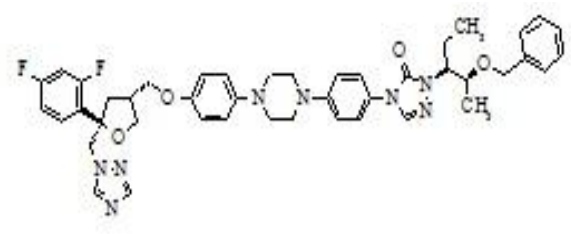

Fig: 4. Chemical structure of TMB.

The proposed method was optimized and validated in accordance with International Conference on Hormonization (ICH) guidelines.[8-10] The aim of present work is to develop a simple, rapid, precise, accurate and selective reversed phase chromatographic method and to estimate the Posaconazole in bulk and its solid dosage forms.

MATERIALS AND METHODS

The reference sample of Posaconazole standard and impurities were supplied as gift sample by spectrum labs, Hyderabad, India and Ajanta Pharmaceutical, Mumbai, India, respectively. All the chemicals were of analytical grade. Acetonitrile (HPLC grade) was used of Merck Pharmaceuticals Private Ltd., Mumbai, India. PHOSPHATE BUFFER used was of HPLC grade and purchased from Loba Chemicals. Commercial Tablets of Posaconazole procured from local market.mg The liquid chromatographic system was of Perkin Elmer (USA), series 200, which consisted of following components: a gradient pump, variable wavelength programmable UV/Vis detector, a manual injection facility with 20 µl fixed loop. The chromatographic analysis was performed using Total Chrom Navigator version 6.3 software on a HiQ Sil C8- 250×4.6 mm, particle size 5µm column.

Preparation of solutions

Buffer preparation

Dissolve 1.36 gr of solid Potasium dihydrogen phosphate in 1000 mL of water.
Adjusted pH to 3.0 ± 0.05 with Phosphoric acid solution.
Filtered through 0.45µm membrane filter.

System suitability

Table 1: system suitability results.

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

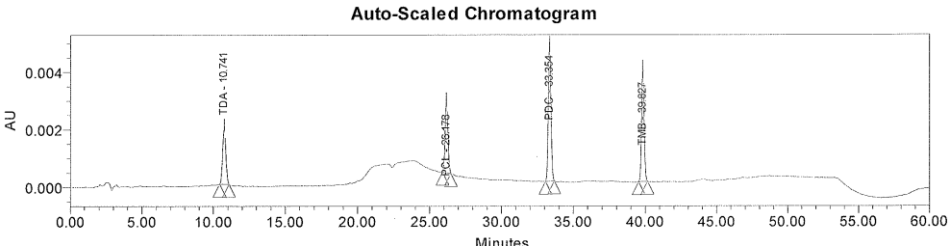

Fig. 5: Chromatogram of system suitability.

Preparation of Solvent A: Buffer.
Preparation of Solvent B: Acetonitrile.

Preparation of Mix solution

Weigh accurately each 5.0 mg of TDA, PDC, TMB and of PCL reference standard into a 5 mL volumetric flask, dissolve and dilute to the volume with diluent and mix.

Buffer preparation

Dissolved 0.77 gm of Ammonium acetate in 1000 mL of water.
Adjusted pH to 6.0 ± 0.05 with Acetic acid.
Filtered through 0.45µm membrane filter.

Preparation of Buffer

Dissolved 1.36 gr of solid Potasium dihydrogen phosphate in 1000 mL of water.
Adjusted pH to 2.5 ± 0.05 with Phosphoric acid solution.
Filtered through 0.45µm membrane filter.

Preparation of Reference stock solution

Weigh accurately each 3.0 mg of TDA, PDC, TMB and 2.0 mg of PCL reference standard into a 100 mL volumetric flask, dissolve and dilute to the volume with diluent and mix.

Preparation of System suitability 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 Reference solution

Take 1.0 mL reference stock solution into a 20 mL volumetric flask, dissolve and dilute to the volume with diluent and mix.

Preparation of Test solution

Weigh accurately about 10.0 mg of test sample into a 10 mL volumetric flask, dissolve and dilute to the volume with diluent and mix**.**

METHODOLOGY

Linearity

Linearity study was conducted for all Known impuritiesand Posaconazole standard in the range of QL level to 150% level. Correlation coefficient values for all impurities were derived from respective linearity graph and the results are given below.

Precision

The precision of the test procedure was evaluated for Posaconazole by injecting the six standard solutions. The Relative Standard Deviation of six injections were calculated. The result of Precision studies is given in Tabulated.

Specificity

Specificity is the ability of a method to discriminate between the analyte (s) of interest and other components that are present in the sample. A study of placebo interference from sexcipients was conducted. Equivalent weight of placebo taken as per the test method and placebo interference was conducted in duplicate 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.

Accuracy

Accuracy of the method was proved by checking the % recovery of each impurity in test solution, spiked with each impurity at QL level, 100% level and 150% level.

Robustness

Robustness of the method is performed by altering the chromatographic conditions such as changing the flow rate, change of temperature, Mobile phase composition and observed the variation of the results which should be within the acceptance criteria.

pH Variation

Posaconazole test sample Spiked with all above impurities was taken and analyzed at two different pH conditions (pH 2.8 and pH 3.2).

Temperature Variation

Posaconazole test sample Spiked with all above impurities was taken and analyzed at two different temperatures (33 ^{0}C and 37 ^{0}C).

Limit of Detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

1. Based on Signal-to-Noise for LOD (3:1), LOQ (10:1)
2. Based on the Standard Deviation of the Response and the Slope

Limit of Quantitation (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

From the linearity data, the limit of detection and quantitation were caliculated using the following formula.

$$LOD = \frac{3.3\,\sigma}{S}, \quad LOQ = \frac{10\,\sigma}{S}$$

S σ = standard deviation of the response, S = slope of the calibration curve.

The QL solution was prepared based on the Limit level solution and obtained the signal to noise ratio between 10 to15.

RESULTS

Linearity

Table 2: 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**	

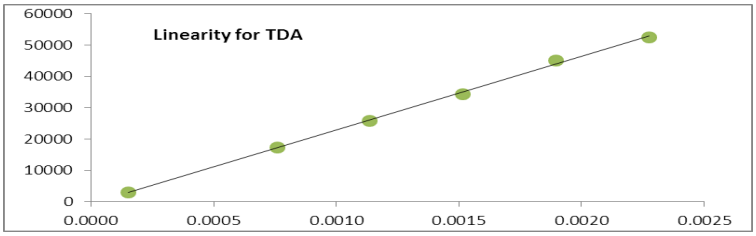

Figure 6: Linearity for TDA.

Table 3: 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**	

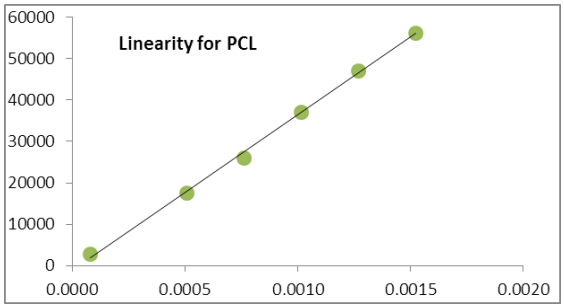

Figure 7: Linearity for PCL.

Table 4: 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**

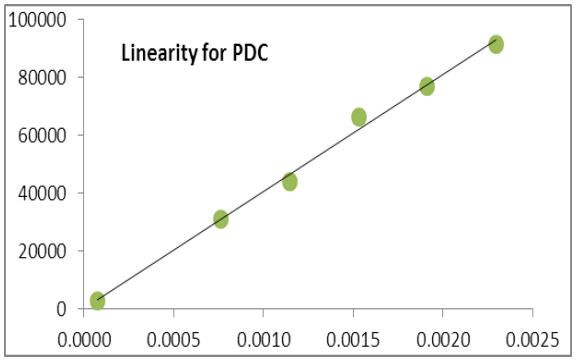

Figure 8: Linearity for PDC.

Table 5: 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**	

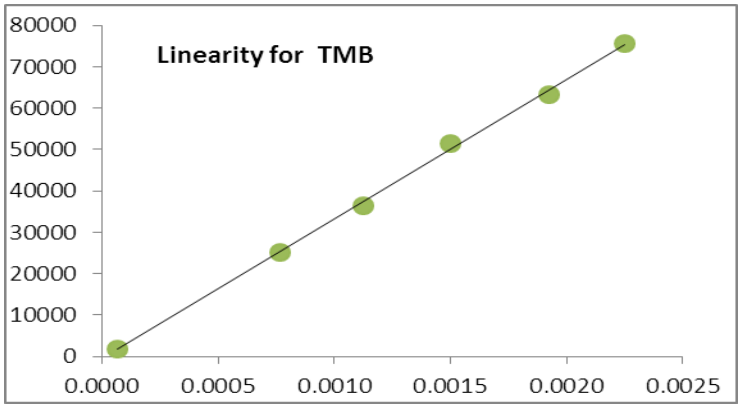

Figure 9: Linearity for TMB.

Precision

Table 6: precision results.

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	

Specificity

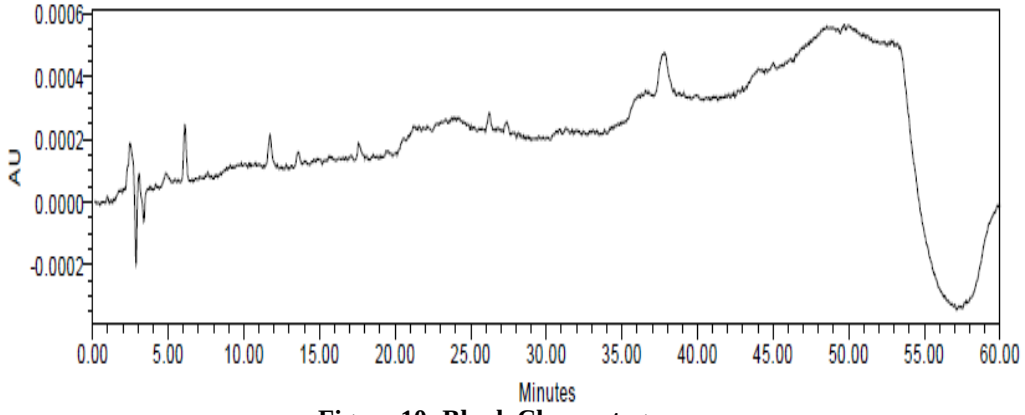

Figure 10: Blank Chromatogram.

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.

Table 7: Retention time of individual solution.

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

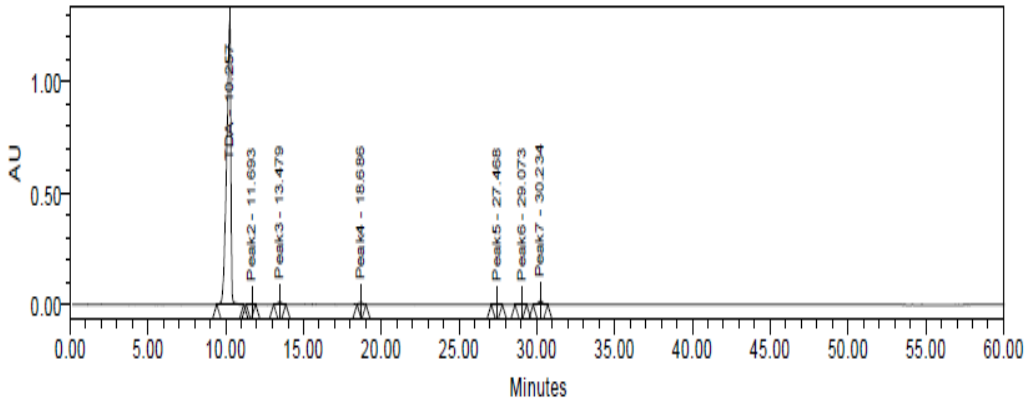

Figure 11: TDA solution chromatogram.

Table 8: TDA solution results.

Peak Results

	RT	Area	Height	% Area	USP Resolution	RT Ratio	Purity1 Angle	Purity1 Threshold	Purity1 Flag	Name
1	10.257	23433531	1278385	96.97			13.076	46.526	No	TDA
2	11.693	46926	2614	0.19	2.63		23.133	21.750	Yes	Peak2
3	13.479	157545	10129	0.65	3.74		7.892	7.131	Yes	Peak3
4	18.686	96191	5767	0.40	12.33		9.588	9.097	Yes	Peak4

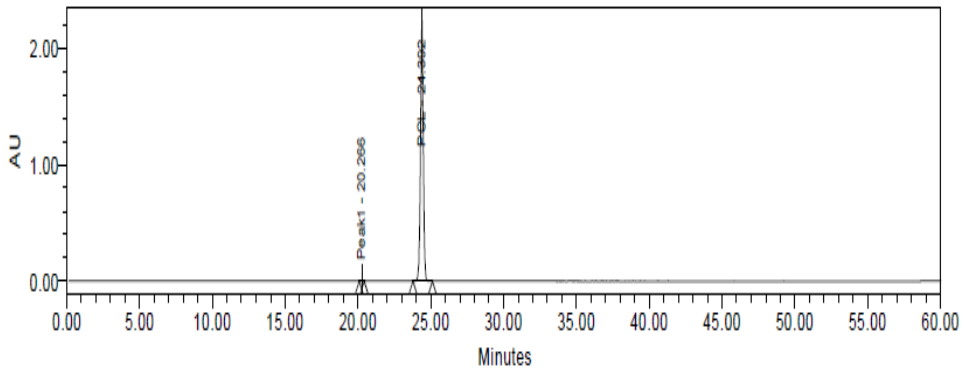

Figure 12: PCL solution chromatogram.

Table 8: Peak Results for PCL solution chromatogram.

Peak Results

	RT	Area	Height	% Area	USP Resolution	RT Ratio	Purity1 Angle	Purity1 Threshold	Purity1 Flag	Name
1	20.266	23243	1670	0.08		0.83	25.232	34.678	No	Peak1
2	24.392	29238160	2237855	99.92	11.54	1.00	13.269	41.882	No	PCL
Sum		29261403								

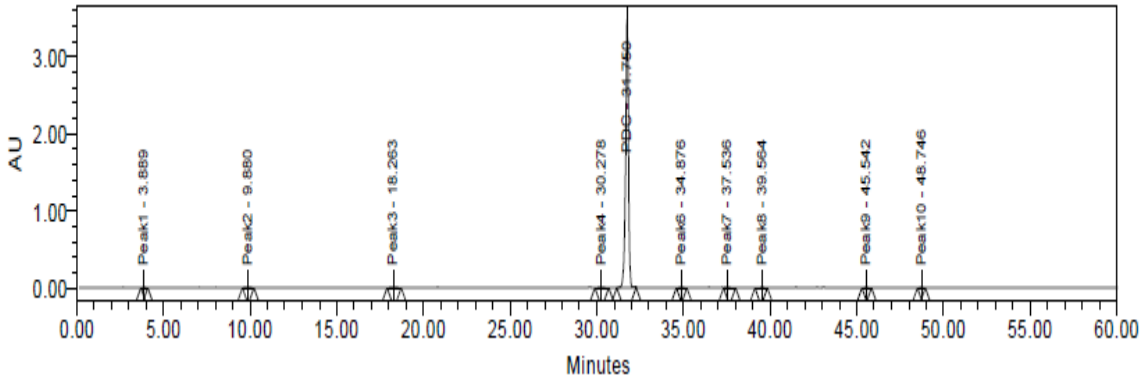

Figure 13: PDC solution chromatogram.

Table 9: Peak Results for PDC solution chromatogram.

Peak Results

	RT	Area	Height	% Area	USP Resolution	RT Ratio	Purity1 Angle	Purity1 Threshold	Purity1 Flag	Name
5	31.750	39904999	3487273	98.32	3.16	1.00	10.804	46.740	No	PDC
6	34.876	56996	4251	0.14	9.63	1.10	14.167	13.995	Yes	Peak6
7	37.536	108330	8720	0.27	7.60	1.18	8.025	7.575	Yes	Peak7
8	39.564	37628	3157	0.09	6.00	1.25	30.259	31.561	No	Peak8
9	45.542	39668	3617	0.10	19.18	1.43	20.596	19.129	Yes	Peak9
10	48.746	38050	3645	0.09	11.51	1.54	21.111	16.445	Yes	Peak10
Sum		40585482								

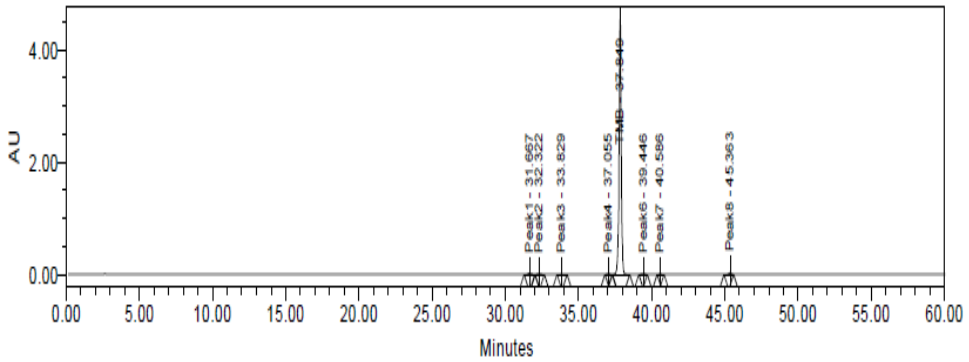

Figure 14: TMB solution chromatogram.

Table 10: TMB solution chromatogram Peak Results.

Peak Results

	RT	Area	Height	% Area	USP Resolution	RT Ratio	Purity1 Angle	Purity1 Threshold	Purity1 Flag	Name
5	37.849	46527788	4558897	97.80	2.62	1.00	13.269	45.754	No	TMB
6	39.446	196203	14942	0.41	5.22	1.04	5.661	5.162	Yes	Peak6
7	40.586	37057	2790	0.08	3.31	1.07	23.631	24.872	No	Peak7
8	45.363	280235	25002	0.59	15.34	1.20	5.106	3.471	Yes	Peak8
Sum		47573345								

Accuracy

Table 11: 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	6.26	3.50	2.46	6.33	3.46	98.40	101.12	99.40
	2.50	6.38	3.50	2.48	6.29	3.48	99.20	98.59	100.20
	2.52	6.23	3.52	2.49	6.46	3.49	98.81	103.69	99.81
100	5.08	12.54	6.08	4.88	12.47	5.88	96.06	99.44	97.06
	5.12	12.56	6.12	4.98	12.79	5.98	97.26	101.83	98.26
	5.06	12.58	6.06	4.76	12.29	5.76	97.07	97.69	98.07
150	7.57	18.89	8.57	7.39	18.79	8.39	97.62	99.47	98.62
	7.73	18.73	8.73	7.68	18.83	8.68	99.35	100.53	100.35
	7.58	18.71	8.58	7.47	18.66	8.47	98.55	99.73	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:

Sample: Posaconazole

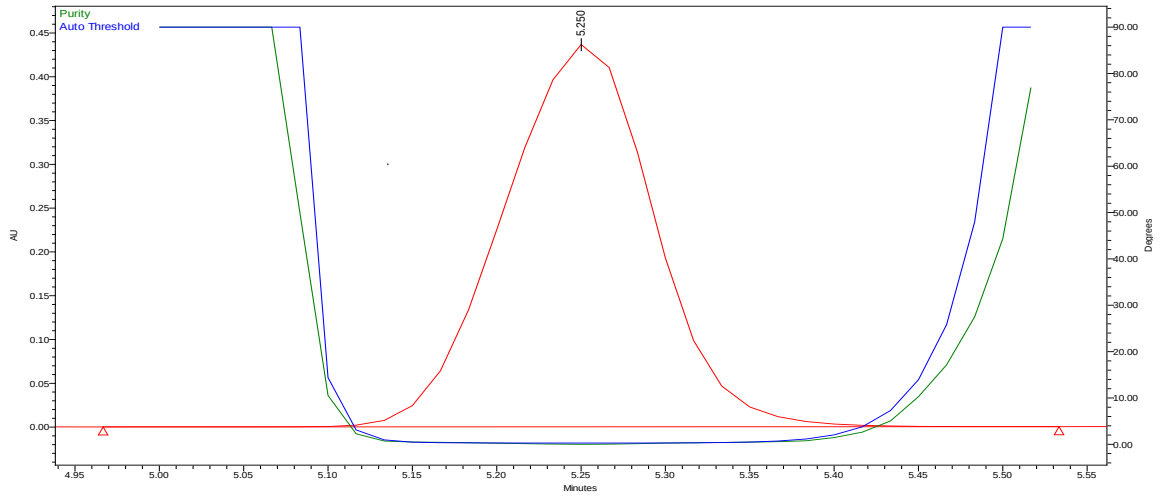

Figure 15: A. Degradation conditions and description of sample:

Purity plot of Posaconazole

Thermal Degradation

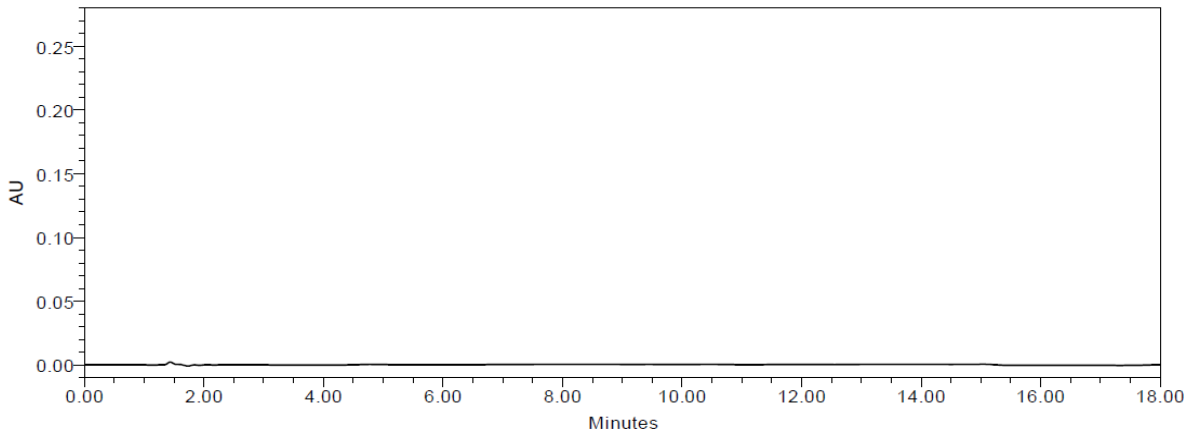

Figure 16: Typical chromatogram of placebo after Thermal degradation.

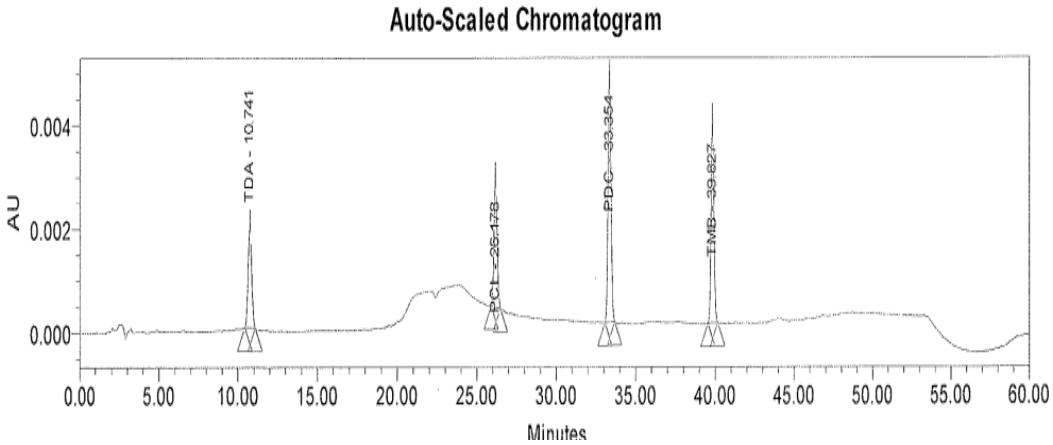

Figure 19: Purity plot of Posaconazole.

Chemical degradation

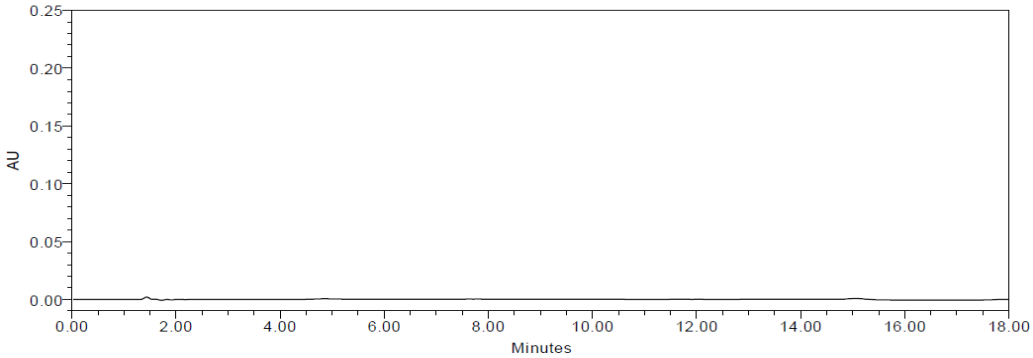

Figure 17: Acid degradation.

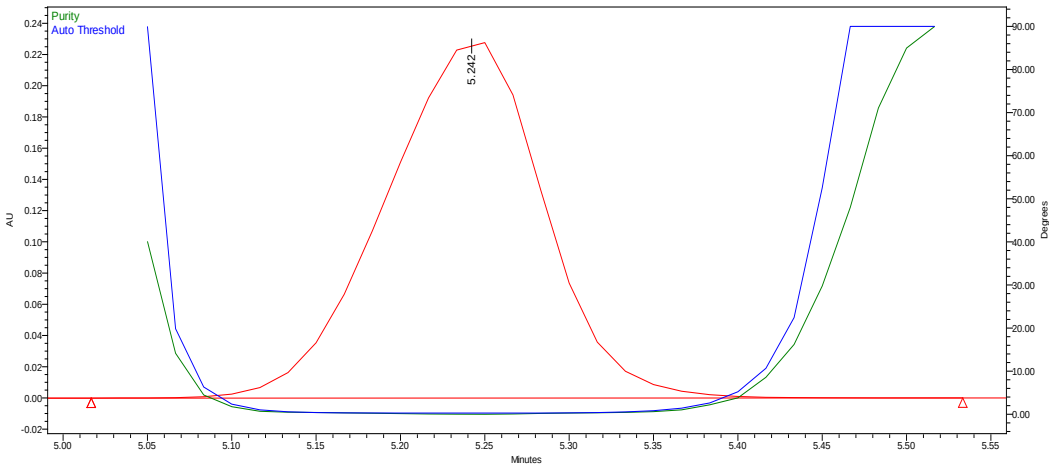

Figure 18: Peak purity plot for Posaconazole in Acid stressed sample

Table 12: 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

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 13: 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

Robustness

Table 14: 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

Temperature Variation

Posaconazole test sample Spiked with all above impurities was taken and analyzed at two different temperatures (33 ^{0}C and 37 ^{0}C).

Table 15: Temperature Variation Results.

Name of the Impurity	Initial 35^{0}C results (%)	33 ^{0}C results (%)	Variation	37 ^{0}C 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.

Table 16: Variation study of Ruggedness (Intermediate precision).

Variation study	Method Precision	Intermediate Precision
Instrument to Instrument	Waters (AD/LC/40)	Waters (AD/LC/85)
Day to Day	23-05-2014	28-05-2014

Table 17: 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

Table: 18 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

RESULTS AND DISCUSSION

Parameters	TDA	PDC	TMB
Tailing factor	1.0	1.06	1.0
%RSD	0.8	0.7	0.9
Theoretical plates	8900	11562	23300
Correlation coefficient	0.9994	0.9975	0.9995
Mean % recovery for 50, 100, 150% respectively	NLT 95.0% NMT 105.0%	NLT 95.0% NMT 105.0%	NLT 95.0% NMT 105.0%
Interference	No interference	No interference	No interference
Flow rate by ± 10%	All the All the system suitability parameters are within the limit for all the variable parameters, for all the three drugs		
Column Oven temperature by ± 5°C			
pH of Buffer solution by ± 0.2 units			
Wavelength of analysis ± 5nm			
Organic composition of mobile phase by ± 5%			
Standard deviation method	5.0 µg/ml	14.9 µg/ml	4.2 µg/ml
	3.1 µg/ml	10.5 µg/ml	13.8 µg/ml

CONCLUSION

In the present investigation, 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.

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