

USING PREPARATIVE CHROMATOGRAPHY AND NMR/LCMS/FT-IR, ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF POSACONAZOLE OXIDATIVE DEGRADATION IMPURITIES

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ABSTRACT

Posaconazole is a pharmacological ingredient that is used to treat fungal infections. It comes in the form of delayed-release tablets. During the forced degradation studies of Tablets, two unknown oxidative degradation impurities were discovered. The impurities were made from Posaconazole using meta-Chloroperoxybenzoic acid as an oxidant in the degradation process. Preparative chromatography was used to separate impurities, and spectroscopic techniques such as IR, NMR, and MS were used to deduce the structures. For the measurement of the total of six associated impurities of Posaconazole in Delayed-Release Tablets, a reverse phase HPLC technique was developed and validated according to ICH Q2 criteria.

Keywords: Posaconazole, Oxidation Degradation Impurities, IR, NMR, and MASS

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INTRODUCTION

Posaconazole is a triazole medication with antifungal properties against *Candida* and *Cryptococcus species*, as well as many *molds* and *endemic fungi*.¹⁻⁶ It's an FDA-approved treatment for oropharyngeal candidiasis, including infections resistant to itraconazole and fluconazole.⁷⁻⁹ The degradation mechanism of Posaconazole in Posaconazole Delayed-Release tablets was investigated using stress research, commonly known as forced degradation.¹⁰ Posaconazole is vulnerable to oxidative circumstances, according to the study, which also found two primary degradation impurities. Because Posaconazole has an Imidazole ring, the impurities are predicted to be Posaconazole N-oxide compounds (Fig.-1). By oxidizing the Posaconazole with meta-Chloroperoxybenzoic acid (mCPBA), the anticipated impurities were prepared.^{11,12} The rate of deterioration was extremely high when H₂O₂ was employed, and Posaconazole was transformed to mono-oxide (s). To manufacture the N-dioxide, mCPBA was used as an oxidant, which allowed for the formation of a di-oxide for any triazole molecule. The contaminants were extracted using column chromatography after oxidation. Chloroform was used to purify the collected fractions. The impurities are N-oxide impurities, one of which is N-dioxide and the other of which is N-mono oxide, which exists in two forms, F-1 and F-2, according to structural elucidation investigations.¹³ Although there is literature on Posaconazole bulk drug assay and related substances, the current study focused on selective oxidation to enrich the N-mono oxides and N-dioxide of Posaconazole, structural elucidation using Fourier-Transform Infrared spectroscopy (FT-IR), Nuclear Magnetic Resonance spectroscopy (NMR), Mass Spectroscopic (MS) techniques. In addition, validation of the developed High-Performance Liquid Chromatography (HPLC) method for Posaconazole degradation and other known contaminants (Fig.-2) according to the International Council for Harmonisation (ICH) Q2(R1) requirements.¹⁴⁻¹⁷

EXPERIMENTAL

All reagents and solvents of chromatographic grade employed for HPLC/LC-MS, a spectroscopic grade for FT-IR, and a high purity grade for NMR studies. In detail, Methanol, Acetonitrile (Merck, Germany, HPLC

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Grade), Water (HPLC Grade from Waters Milli-Q system), Dipotassium hydrogen phosphate anhydrous (Merck, Emparta), Orthophosphoric acid (Merck, ACS), Hydrochloric Acid (HCl) (36% w/w) (Merck, GR Grade), Sodium hydroxide (NaOH) pellets (Merck, Emparta), Hydrogen peroxide (H₂O₂) 30% (Merck, AR Grade), Magnesium Oxide (Merck, Emparta), Dimethyl sulfoxide- d₆ (Sigma, Spectroscopic Grade), Potassium Bromide (Rankem, Spectroscopic Grade), meta- Chloroperoxybenzoic acid (Sigma Aldric, Reagent Grade), Sodium Chloride (Merck, Empire) and Dichloromethane (Merck, HPLC Grade). Posaconazole delayed-release tablets manufactured by Slayback Pharma, Noxafil tablets manufactured by Merck, Posaconazole drug substance manufactured by MSN laboratories. Impurities namely Tosylated compound, Hydroxytriazole, Deshydroxy-Posaconazole (Deshydroxy), Benzylated-Posaconazole (Benzylated) manufactured by MSN laboratories.

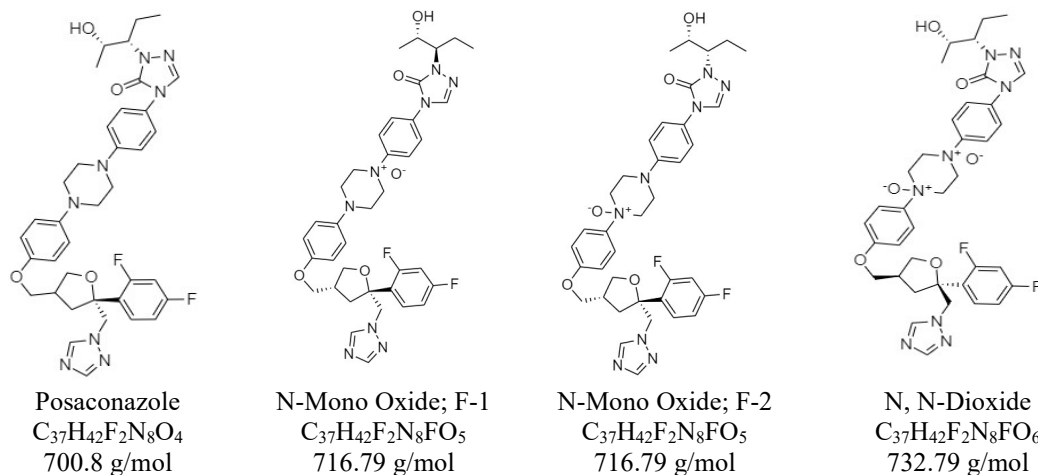


Fig.-1: Chemical Structure of Posaconazole and N-Oxide's

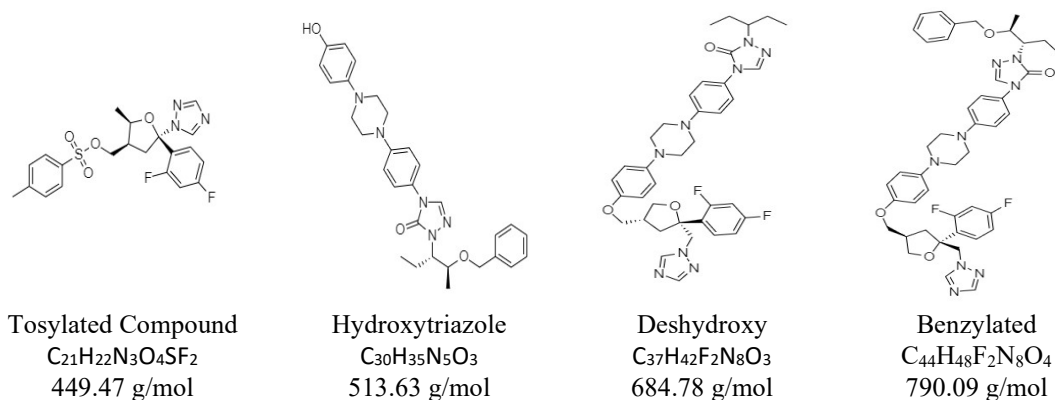


Fig.-2: Chemical Structure of other Known Impurities

Solutions Preparations

Standard solution; Prepared a posaconazole standard solution containing 4.0ppm (parts per million) in diluent (a 40:60 v/v mixture of water and acetonitrile). **Sample solution:** weigh and finely powder not less than ten tablets, transfer equivalent to 200 mg of posaconazole from fine powder into a 100 mL volumetric flask, add 70 mL of diluent, sonicate for 30 minutes and magnetic stir for 60 minutes, then dilute to volume with diluent.

Degradation Conditions

Acid; 5 mL 5N hydrochloric acid was added to the sample solution and heated in a water bath for 6 hours at 60°C, after which the sample was neutralized with 5 mL 5N sodium hydroxide. **Base;** 5 mL 5N sodium hydroxide was added to the sample solution and heated in a water bath for 6 hours at 60°C, after which the sample was neutralized with 5 mL 5N hydrochloric acid. **Oxidative;** 5 mL of 3 percent H₂O₂ was added to

the sample solution and heated for 6 hours at 60°C in a water bath. *Thermal*; heated tablet powder for 24 hours at 80°C in a dry oven. *Humidity*; Tablet powder was stored in a humidity chamber with a relative humidity of 90% for roughly 7 days. *Photolytic*; tablet powder was subjected to light with “a total illumination of at least 1.2 million lux hours and an integrated near ultraviolet energy of at least 200-watt hours/square meter.”

Sample Preparation Procedure for Impurities Isolation and Purification Process

In a 100 mL round bottom flask, 3.0 g of Posaconazole medication ingredient is added, along with 50 mL of dichloromethane (DCM), and thoroughly mixed. Cooled the reaction mass for 10 minutes at -15°C to -20°C before adding 2.5 g of mCPBA reagent. For 30 minutes, the reaction mixture was stirred at the same temperature. Filtered the contents and rinsed them twice with 1N sodium hydroxide solution, then brain solution (saturated sodium chloride solution), then 6N hydrochloric acid. Using a separating funnel, separated the DCM layer and distilled it; the approximate yield is 1.0 g. Using a preparative column chromatographic method, mixed N-oxide compounds were further separated. N-mono oxide and a pure form of N-dioxide (with a 92:8 mix ratio of forms F-1 and F-2) were isolated.

Instrumentation

HPLC Conditions

An Agilent HPLC (Agilent Technologies, Germany) system equipped with a quaternary pump, column heater, sample cooler, and diode array detector has been used. Mobile phase comprising in combinations of a 25mM dipotassium hydrogen phosphate pH 8.1 buffer (4.3 g dipotassium hydrogen phosphate salt dissolved in 1000 mL water, pH adjusted to 8.1 with orthophosphoric acid) and methanol in the ratio of 75:25 v/v is one of the mobile phase components (A) and Acetonitrile as (B) with a gradient program of, from 0.0-30.0 min 65:35 (v/v), a change in composition to 38:62 from 30.0 min to 58.0 min, isocratic with the ratio of 38:62 till 65.0 min and from 66.0 to 75.0 the initial gradient was pumped with a flow rate of 1.3 mL/min. With a column-controlled temperature of 35°C, the reliable separation was obtained on a waters X-Bridge C18 250 mm X 4.6 mm, 5m column (Waters, Part No.:186003117). With a PDA (Photo Diode Array) detector, the eluent was measured at 262 nm. Each sample was injected into HPLC at a volume of 5µL, and the data was processed using the Agilent CDS -Version 2.3 software controller.

Preparative Liquid Chromatography

Using the above sample, isolation of degrading impurity was performed in an Agilent preparative liquid chromatography system with a flow rate of 4 mL/min using the method described under section HPLC conditions. For chromatographic separations, an X-Bridge OBD C18 preparative column (250 mm X 19 mm, 5µm; SKU: 186004021) was employed, with the eluent monitored at 262 nm. Compounds were extracted to dichloromethane and fractions were collected based on retention durations.

Mass Spectroscopy

In acetonitrile, a 300ppm solution for each chemical was produced, collected into a vial, and placed in the autosampler. The spectra were acquired when a little amount of sample was introduced into the LC-MS (Make: Shimadzu).

FTIR Spectroscopy

A little amount of the separated compound was placed in the FTIR instrument's crystal region. Background spectral noise was removed from the spectra before recording. With eight scans, the spectra were recorded in the range of 4000 1/cm to 450 1/cm (Make: Perkin Elmer).

NMR Spectroscopy

A little amount of each isolated compound was mixed in deuterated Chloroform, together with a small amount of tetramethylsilane reference material, and the mixture was filtered through a glass wool plug using a ball of dried glass wool. The sample was transferred to a sample tube, and the tube was inserted in the spinner, which was then placed in the depth gauge. In the NMR apparatus, scanned the samples for spectra (Make: Bruker).

RESULTS AND DISCUSSION

Detection of Degradation Impurities by HPLC

The N-oxide impurities acquired in the isolation procedure exhibited the same chromatographic behavior as impurities found in the peroxide stress investigation of Posaconazole delayed released tablets, as seen in chromatograms (Fig.-3). Individual solutions had retention times of 2.784 minutes (Posaconazole N-dioxide) and 4.430 minutes (Posaconazole N-dioxide), which corresponded to the retention times of peaks in degradation samples of 2.762 minutes and 4.411 minutes, respectively.

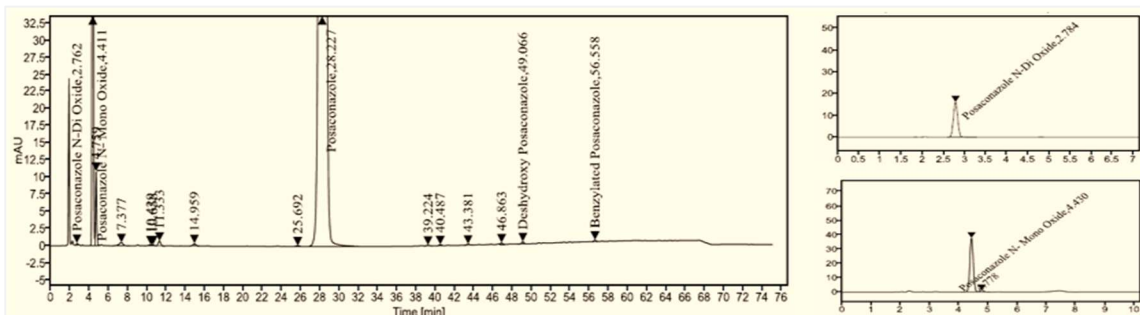


Fig.-3: Chromatogram's of Peroxide degradation sample and N-oxide(s)

IR

The primary absorption bands (stretching) in the infrared spectra of both N-oxide compounds suggested the functional groups -O-H, Aromatic -C=C, -C=O, C-O (Ether), C-H, -C-F, Aromatic C-H, and C-O (Alcohol). The spectral signals and their interpretation (Table-1) match the hypothesized structural formulas for Posaconazole N-Oxide impurities. (Fig.-4a and Fig.-4b).

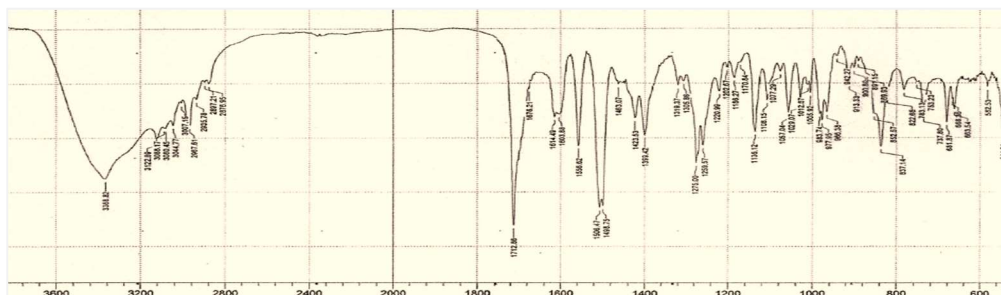


Fig.-4a: IR Spectra of N-dioxide

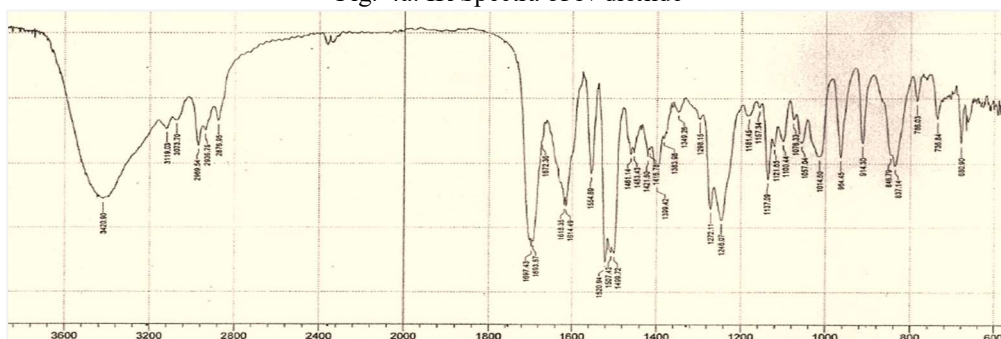


Fig.-4b: IR Spectra of N-mono oxide

Table-1: IR Spectral Data of Posaconazole N-Oxide Impurities

No	Wavenumber (1/cm)		Proposed Functional Group	Mode of Vibration
	N-dioxide	N-monooxide		
1	3368.82	3420.90	-O-H	Stretching
2	1506.47, 1614.49	1520.94, 1618.35	Aromatic -C=C	Stretching

3	1712.86	1697.43	-C=O	Stretching
4	1258.57	1248.07	C-O (Ether)	Stretching
5	2967.61	2959.54	C-H	Stretching
6	1399.42	1399.42	-C-F	Stretching
7	3083.17	3073.70	Aromatic C-H	Stretching
8	1136.12	1137.09	C-O (Alcohol)	Stretching

NMR

The ^1H NMR chemical shifts of Posaconazole N-dioxide and N-mono oxide were assigned to functional groups, which supports the hypothesized molecular structures (Table-2) (Fig.-5a and Fig.-5b).

Table-2: NMR Spectral Data of Posaconazole N-Oxide Impurities

No	Chemical Shift value ' δ '		No of Protons	Multiplicity
	N-dioxide	N-mono oxide		
1	0.740-0.776	0.724-0.762	3	triplet
2	1.132-1.144	1.116-1.128	3	doublet
3	1.704-1.739	1.693-1.726	4	triplet
4	2.159-2.618	2.143-2.601	2	multiplet
5	3.020-3.070	3.073-3.096	5	triplet
6	3.334	3.317	4	singlet
7	3.759-3.917	3.598-3.887	2 and OH	multiplet
8	4.033-4.072	4.017-4.137	3	triplet
9	4.551-4.926	4.543-4.679	2	multiplet
10	7.001-7.316	6.820-7.567	10	multiplet
11	7.909-7.931	7.868-7.890	1	doublet
12	8.082-8.104	8.049-8.071	1	doublet
13	8.569	8.558	1	singlet

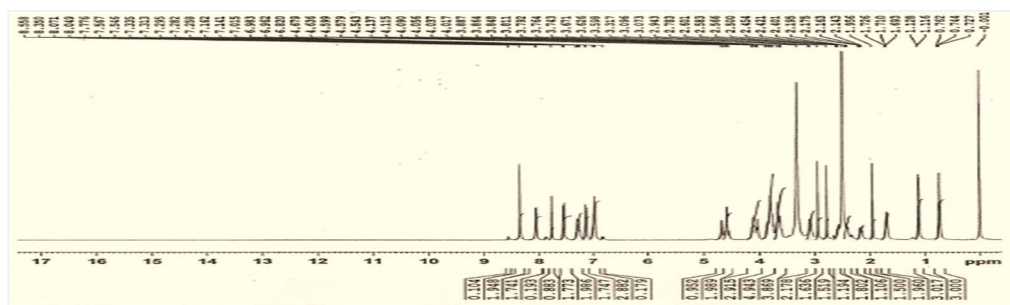


Fig.-5a: NMR Spectrum of N-dioxide

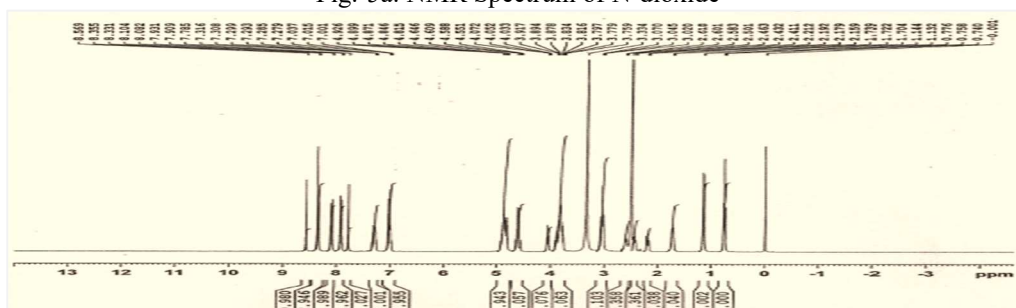


Fig.-5b: NMR Spectrum of N- mono oxide (s)

MS

The molecular ion peak of N-dioxide impurity is 733.2 ($M+1$) and the molecular ion peak of N-mono oxide impurity is 717.2 ($M+1$) according to mass spectroscopy data (Fig.-6a and Fig.-6b). The hypothesized

molecular formulae of N-dioxide, $C_{37}H_{42}F_2N_8O_6$ with a molecular weight of 732.79 g/mol, and N-mono oxide, $C_{37}H_{42}F_2N_8O_5$ with a molecular weight of 716.79 g/mol were supported by the molecular ions data.

Method Validation

For Posaconazole and its related impurities, the improved HPLC technique was verified according to ICH Q2 (R1) requirements. All parameters were assessed, including specificity, system appropriateness, LOD and LOQ, accuracy, precision, robustness, linearity, and solution stability. Specificity chromatograms for spiked samples are depicted below (Fig.-7a and Fig.-7b), and the results are summarized in Tables-3 to 9.

Table-3: Forced Degradation Data

Degradation Details (% Net Degradation)						
Name	Acid	Alkali	Oxidative	Photolytic	Thermal	Humidity
Assay (%) ^a	94.7	98.6	60.9	93.1	98.1	99.5
Net degradation (%) ^a	1.3	0.2	35.9	3.3	0.3	0.1
Mass balance ^{a, b}	96	99	96	96	98	100
Purity factor ^a	1.000	1.000	1.000	1.000	1.000	1.000
Purity Check ^a	Pass	Pass	Pass	Pass	Pass	Pass

^a for the Posaconazole peak; ^b Mass balance = % Assay + % Net Degradation.

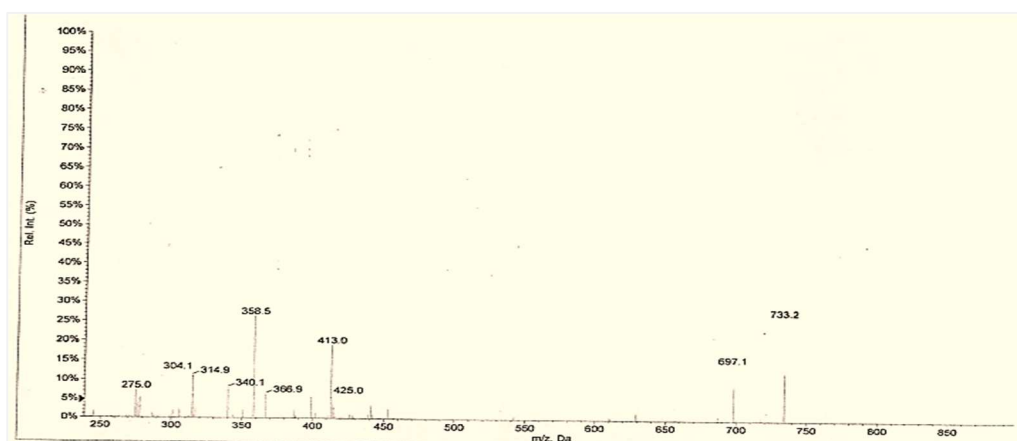


Fig.-6a: Mass Spectrum of N-dioxide

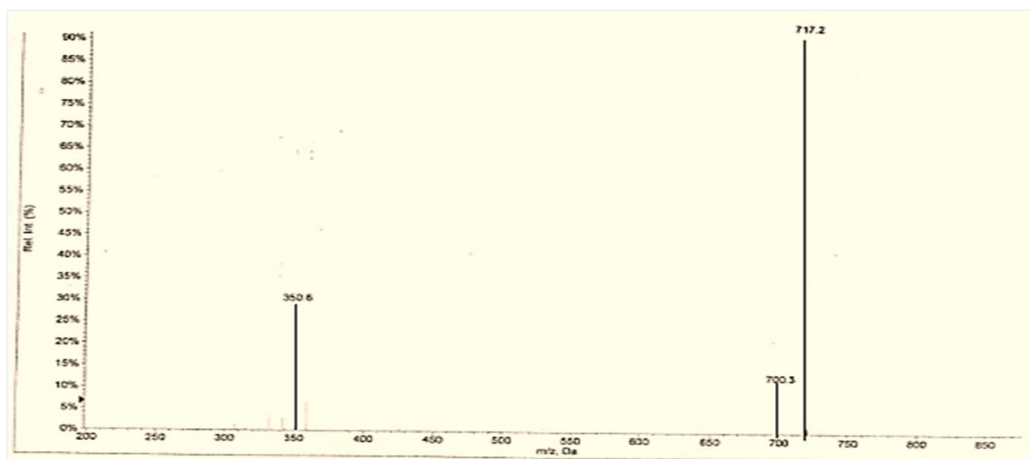


Fig.-6b: Mass Spectrum of N- mono oxide (s)

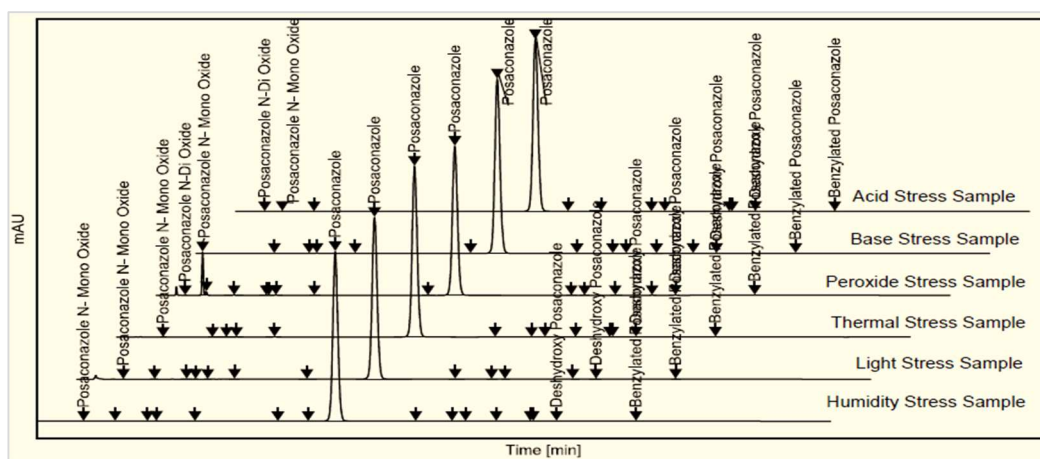


Fig.-7a: Overlaid Chromatogram of Stress Study Samples

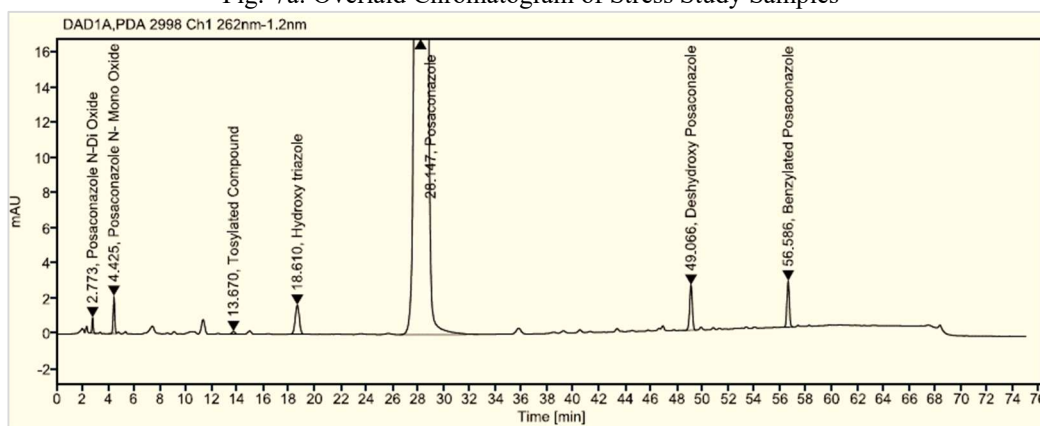


Fig.-7b: Chromatogram of the Spiked Sample

Table-4: Stress Testing – All detected Impurities List

Degradation Details (% Individual Impurities)						
Name	Acid	Alkali	Oxidative	Photolytic	Thermal	Humidity
N-dioxide	ND ^a	ND	2.1	ND	ND	ND
N-mono oxide	0.01	0.02	25.3	0.01	0.03	ND
Tosylated Compound	ND	ND	ND	ND	ND	ND
Hydroxy Triazole	ND	ND	ND	ND	ND	ND
Deshydroxy Posaconazole	0.05	0.05	0.05	0.05	0.05	0.05
Benzylated Posaconazole	0.03	0.03	0.03	0.03	0.03	0.03

^a ND- Not Detected

Table-5: Summarized Data of Method Validation Study: RT, RRT, LOD, LOQ, LOQ Precision, and LOQ Accuracy

Name	RT (Min)	RRT	LOD and LOQ				LOQ Precision	LOQ Recovery
			LOD Con (µg/mL) ^a	S/N	LOQ Con (µg/mL) ^b	S/N	% RSD ^c	n=3; % Average
N-dioxide	2.77	0.10	0.2	10	0.4	29	2.3	94.2
N-mono oxide	4.42	0.16	0.2	20	0.4	48	3.1	91.4
Tosylated Compound	13.67	0.49	0.2	4	0.4	19	1.9	97.8
Hydroxy Triazole	18.61	0.66	0.2	5	0.4	21	9.1	102.1
Posaconazole	28.15	1.00	0.2	7	0.4	13	3.4	102.0

Deshydroxy Posaconazole	49.07	1.74	0.2	12	0.4	29	5.9	100.1
Benzylated Posaconazole	56.59	2.01	0.2	16	0.4	30	1.9	99.1

^a LOD conc.: Limit of detection concentration; ^b LOQ conc.: Limit of quantification concentration;

^c RSD = relative standard deviation, n: number of samples prepared; S/N = Signal to Noise ratio;

RT = Retention Time; RRT = Relative Retention Time

Table-6: Summarized Data of Method Validation Study: Accuracy and Precision

% Recovery at Accuracy Levels				% RSD	
Name	50%; n=3 (2.0 µg/mL)	100%; n=6 (4.0 µg/mL)	150%; n=3 (6.0 µg/mL)	Method Precision	Intermediate Precision
N-dioxide	98.6	97.5	96.5	1.8	2.3
N-mono oxide	92.1	95.3	93.8	3.1	2.1
Tosylated Compound	98.1	97.8	97.4	2.9	0.8
Hydroxy Triazole	93.3	93.4	95.5	0.9	1.6
Posaconazole	97.0	98.0	97.0	0.5	0.2
Deshydroxy Posaconazole	96.1	95.6	97.4	0.6	1.5
Benzylated Posaconazole	94.9	93.5	95.8	0.5	1.9

RSD = relative standard deviation for six samples; n: number of samples prepared.

Table-7: Summarized Data of Method Validation Study: Robustness

Robustness										
Parameter	LF	HF	LCT	HCT	LPH	HPH	LO	HO	LBS	HBS
Retention Time in min	31.6	23.2	29.0	24.5	29.4	28.6	33.5	29.5	30.9	30.1
The USP tailing factor ^a	1.03	1.02	1.04	1.00	1.04	1.03	0.98	0.99	0.99	1.01
% RSD (n=3) ^{a, b}	0.2	0.7	0.4	0.7	0.3	0.7	0.8	1.4	1.0	1.9
USP Plate count ^a	16757	14462	13158	15492	13988	16050	21743	15063	16091	16651

^a = for Posaconazole peak; NMT= Not more than; NLT= Not less than; ^b = relative standard deviation;

n= standard replicate injections; LF = Low Flow; HF = High Flow; LCT = Low Column Temperature;

HCT = High Column Temperature; LPH = Low pH; HPH = High pH; LO = Low Organic; HO = High Organic; LBS = Low Buffer Strength; HBS = High Buffer Strength

Table-8: Summarized Data of Method Validation Study: System Suitability

No	Parameter	Acceptance Criteria	Observed Value
1	The USP tailing factor ^a	NMT 2.0	1.00
2	% Relative standard deviation (n=3) ^a	NMT 10.0	0.9
3	USP Plate count ^a	NLT 2000	16358

^a = for Posaconazole peak; NMT= Not more than; NLT= Not less than;

n= standard replicate injections

Table-9: Summarized Data of Method Validation Study: Solution Stability

No	Solution Stability		
	Solution name	Duration in Days	Condition
1	Standard solution	4	Room temperature ^a
2	Standard solution	4	Refrigerated Condition ^b
3	Sample solution	3	Room temperature
4	Sample solution	3	Refrigerated Condition
5	Mobile Phase	4	Room Temperature

^a25°C; ^b2-8°C

CONCLUSION

The degradation experiments of Posaconazole Delayed-release tables revealed that Posaconazole was only degraded in oxidative circumstances and that Posaconazole remained stable in all other conditions. N-oxides of Posaconazole were proven as important oxidative degradants in structure elucidation studies. One of the recognized N-oxides is N-dioxide, and the other is N-mono oxide, which comes in two forms: F-1 and F-2. The most prominent form among n-mono oxides, according to structural elucidation data, is F-2. According to ICH Q2(R1), the analysis method was validated for determining associated impurities of Posaconazole in delayed-release tablets, and it was discovered that the method is stability indicative and can be utilized for its intended purpose.

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