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LC–UV and LC–MS evaluation of stress degradation behaviour of tenatoprazole

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ABSTRACT

In the present study, comprehensive stress testing of tenatoprazole was carried out according to ICH guideline Q1A (R2). Tenatoprazole was subjected to stress conditions of hydrolysis, oxidation, photolysis and neutral decomposition. Additionally, the solid drug was subjected to 50 °C for 60 days in dry-bath, and to the combined effect of temperature and humidity at 40 °C/75% RH. Extensive degradation was found to occur in acidic, neutral and oxidative conditions. Mild degradation was observed in basic conditions. The drug is relatively stable in the solid-state. The products formed under different stress conditions were investigated by LC and LC–MS. Successful separation of drug from degradation products formed under stress conditions was achieved on a Chemito ODS-3 column [C_{18} (5 μm, 250 mm × 4.6 mm, i.d.)] using methanol: 0.01 M acetate buffer pH 4.5 adjusted with glacial acetic acid (55:45) as the mobile phase at flow rate of 1 mL/min and the peak was detected using a UV detector set at 306 nm. The LC–MS *m/z* values and fragmentation patterns of degradation products formed under different stress conditions were studied and characterized through LC–MS fragmentation. Based on the results, degradation pathway for drug has been proposed.

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1. Introduction

Tenatoprazole is a novel proton pump inhibitor which has imidazopyridine ring connected to a pyridine ring by sulfinyl-methyl chain. Tenatoprazole (Fig. 1), 5-methoxy-2-(3,5-dimethyl-4-methoxy)-2-pyridyl]methylthio]-imidazole[4,5-b]pyridine is a prodrug of the proton pump inhibitor (PPI) class, which is converted to the active sulfenamide or sulfenic acid by acid in the secretory canaliculus of the stimulated parietal cell of the stomach. This active species binds to luminally accessible cysteines of the gastric H^+, K^+-ATPase resulting in disulfide formation and acid secretion inhibition [1,2]. However, the anti-secretory and anti-ulcer effects of tenatoprazole were reported to be 2–4 times more potent than those of omeprazole with long-lasting effects on gastric acid secretion [3]. All proton pump inhibitors are unstable when exposed to an acidic milieu, such as the stomach. Therefore, they are formulated with an enteric coating that shields the active drug from the acidic gastric environment [4,5]. Tenatoprazole has a greatly extended plasma half-life in comparison with other proton pump inhibitors [6]. There is a dearth of analytical methods reported in the literature. HPLC method for the quantitative determination of tenatoprazole in rat plasma [7], pharmacokinetic study in dog plasma [2,8] and pharmacokinetic study in healthy male Caucasian volunteers [9] have been reported. These methods were developed for the purpose of determining low level of drug substance in the biological samples, thus they are not suitable for routine analysis of formulated product where the content of API is high in the formulation.

The parent drug stability test guideline Q1A (R2) issued by the International Conference on Harmonization (ICH) [10] suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to identification of degradation products and, hence, better understanding the stability of the drug molecule. It also requires that analytical test procedures for stability samples should be stability-indicating and should be fully validated [11].

A comprehensive LC and LC–MS study of the degradation behaviour of tenatoprazole under various ICH prescribed stress condition has been lacking. So we decided to carry out forced decomposition studies according to the ICH requirements and develop a selective and validated stability-indicating HPLC method. An integral aim of the study was to identify degradation products, and to postulate complete degradation pathway of the drug.

2. Experimental

2.1. Materials

Dr. Reddy's Laboratories Ltd. (Hyderabad, Andhra Pradesh, India), kindly supplied pure drug sample of Tenatoprazole as a gift sample of Batch No.: 3B060789. It was used without further

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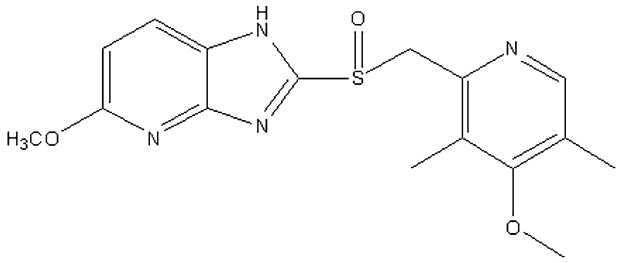

Fig. 1. Structure of tenatoprazole.

purification and certified to contain 96.5% (w/w) on dried basis. Twenty tablets of tenatoprazole – 20 mg were procured as gift sample from Dr. Reddy's Laboratories Ltd., Hyderabad, Andhra Pradesh, India. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, Mumbai, Maharashtra, India.

2.2. Instrumentation

The LC system consisted of a Pump (model Jasco PU 2080); Intelligent LC pump with sampler programmed at 20 μL capacity per injection was used. The detector consisted of a UV/VIS (Jasco UV 2075) model operated at a wavelength of 306 nm. Data was integrated using Jasco Borwin version 1.5, LC–Net II/ADC system. The column used was Chemito ODS-3–C_{18} (250 mm × 4.6 mm, 5.0 μm) from Berlin, Germany.

LC–MS studies were carried out on a system in which LC part consisted of 1100 series HPLC (Agilent Technologies, Wald-bronn, Germany) comprising of an on-line degasser (G1379A), binary pump (G131A), auto injector (G1313A), column oven (G1316A) and PDA detector (G1315B).

The MS system consisted of QSTAR® Pulsar API Hybrid Q-TOF tandem mass Spectrometer (Applied Biosystems-PE Sciex, USA). The mass spectras of tenatoprazole and the degradation products were taken in ESI (Turbo spray) positive mode in mass range of 100–500 and analyzed in the triple quadrapole analyzer. Samples were dissolved in methanol in a concentration range of 10 μg/mL and injected into the inlet. The whole process was controlled using an Analyst® Q5 software installed in a Dell Precision 530 Workstation. The LC system attached to the MS was a High Throughput HPLC–LC-2010HT (Shimadzu Corp., Japan); mobile phase used was methanol: 0.01 M acetate buffer pH 4.5 adjusted with acetic acid (55:45) and column used was Chemito ODS-3–C_{18} (250 mm × 4.6 mm, 5.0 μm) from Berlin, Germany. Flow rate was kept at 1 mL/min eluent from column was introduced in to MS through flow splitter which splits volume of mobile phase and deliver minimum amount of mobile phase in MS. The split ratio was 1:1.

2.3. Forced degradation studies

A stock solution containing 100 mg tenatoprazole in 100 mL methanol was prepared. This solution was used for forced degradation to provide an indication of the stability-indicating property and specificity of proposed method. In all degradation studies the average peak area of tenatoprazole after application (10 μg/mL) of seven replicates was obtained.

2.3.1. Acid and base induced degradation

Acid decomposition studies were performed by refluxing the solution of drug (1 mg/mL) in 0.01 M hydrochloric acid at room temperature for 30 min. The studies in alkaline condition were carried out in 1 M sodium hydroxide and the solution of drug (1 mg/mL) was refluxed for 4 h at 80 °C. The resultant solutions were first neutralized to prevent secondary decomposition in the sample prep or on-column as some of the degradation is rapid and diluted with methanol to obtain 10 μg/mL solutions and 20 μL were injected into the system.

2.3.2. Hydrogen peroxide induced degradation

To study hydrogen peroxide induced degradation, the drug solution (1 mg/mL) was exposed to 6% hydrogen peroxide at room temperature for a period of 30 min, and then heated in boiling water bath for 10 min to completely remove the excess of hydrogen peroxide. The resultant solutions were diluted to obtain 10 μg/mL solutions and 20 μL were injected into the system.

2.3.3. Photochemical degradation

The photochemical stability of the drug was studied by exposing the stock solution of drug (1000 μg/mL) as well as solid drug using quartz container to direct sunlight for 1 h on a wooden plank and kept on terrace. The solution was diluted with methanol to obtain a solution of 10 μg/mL and then 20 μL of the solution was injected into the system.

2.3.4. Dry heat and wet heat degradation

The standard drug in solid form was placed in oven at 50 °C for 60 days to study dry heat degradation and for wet heat degradation drug was kept in humidity chamber at 50 °C, 75% relative humidity (RH) for 3 months.

2.4. Optimization of stability-indicating HPLC method

The HPLC procedure was optimized with a view to develop stability-indicating assay method. Pure drug along with its degraded products were injected and run in different solvent systems. Initially methanol and water in different ratios were tried. It was found that when methanol concentration was increased in the mobile phase, the degradation product started to elute in dead volume. Hence concentration of methanol was decreased and there was improvement in resolution. It was found that methanol: 0.01 M acetate buffer pH 4.5 adjusted with acetic acid (55:45) as a mobile phase at a flow rate of 1 mL/min gives acceptable retention time (t_R), theoretical plates and good resolution of drug and degradation products.

2.5. Validation of the method

Validation of optimized LC method was done with respect to following parameters.

2.5.1. Linearity and range

Linearity of the method was studied by injecting seven concentrations of the drug prepared in the mobile phase in the range of 0.1–10 μg/mL in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

2.5.2. Precision

Precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations (0.5, 2.5, 5 μg/mL) of the drug in hexaplicate ($n = 6$) on the same day. Intermediate precision of the method was checked by repeating studies on three different days. Additionally, the developed HPLC method was checked through separation studies on the mixture of reaction solutions on a different chromatographic system on a different day.

2.5.3. Limit of detection and limit of quantitaiton

The signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1.The LOD and LOQ were experimentally verified by diluting known concentrations of standard solution of tenatoprazole until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

2.5.4. Robustness of the method

To evaluate robustness of the HPLC method, few parameters were deliberately varied. The parameters included variation of flow rate, percentage of methanol in the mobile phase, pH of mobile phase. The resolution of drug in a mixture of stressed samples was studied by performing the analysis on a different chromatographic system. Robustness of the method was done at three different concentration levels 0.5, 2.5, 5 μg/mL for tenatoprazole. Also robustness was verified by studying the resolution of drug in a mixture of degraded samples on different chromatographic system on different days.

2.5.5. Specificity

The specificity of the method towards the drug was established through study of resolution factor of the drug peak from the nearest resolving peak. Overall selectivity was established through determination of purity for each degradation product peak using PDA detector.

2.5.6. Accuracy

Accuracy of the developed method was tested by fortifying a mixture of decomposed reaction solutions with three concentrations of drug corresponding to 80%, 100% and 120% and determining the recovery of added drug. At each level of the amount six determinations were performed. Also recovery studies were carried out by applying the method to drug sample to which known amount of tenatoprazole corresponding to 80%, 100% and 120% of label claim had been added (standard addition method).

2.6. Analysis of marketed formulation

To determine the content of tenatoprazole in enteric coated tablets labeled to contain 20 mg of drug procured from Dr. Reddy's Laboratories Ltd., Hyderabad, India, 20 tablets were weighed, their mean weight determined and they were finely powdered and powder equivalent to 20 mg tenatoprazole was weighed. This was transferred into a 100 mL volumetric flask containing 50 mL methanol, sonicated for 30 min and diluted to 100 mL with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min. Supernatant was taken and after suitable dilution the sample solution was then filtered using 0.45 μm filter (Millipore, Milford, MA). The above stock solution was further diluted to get sample solution at three different concentrations of 80, 100 and 120 μg/mL, respectively. A 20 μL volume of each sample solution was injected into LC, six times, under the conditions described above. The peak areas were measured at 306 nm and concentrations in the samples were determined using multilevel calibration developed on the same LC system under the same conditions using linear regression equation.

2.7. Development of LC–MS method and characterization of degradation products

To characterize degradation products by LC–MS studies, the developed method was used without modification. Satisfactory

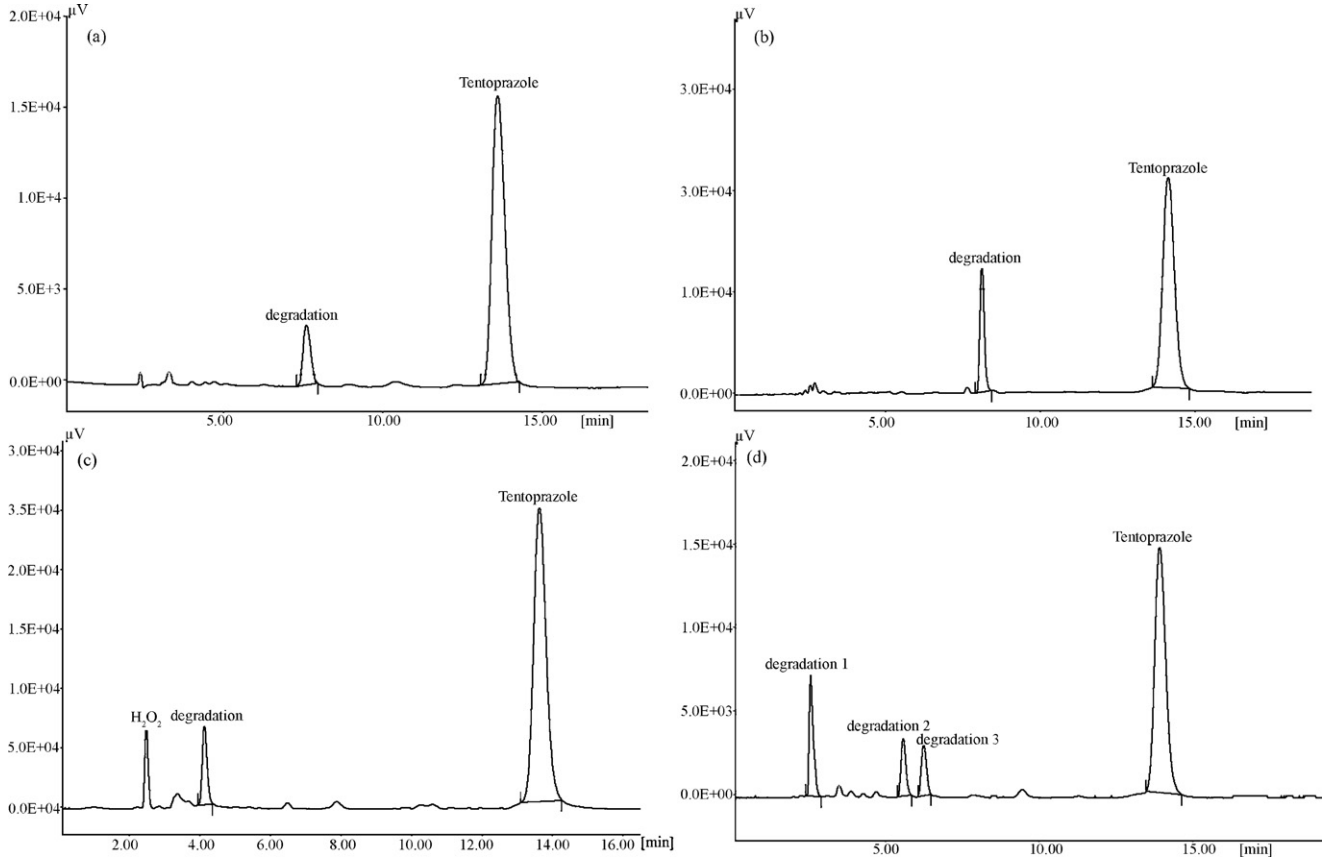

Fig. 2. Representative HPLC chromatograms of tenatoprazole. (a) Sample degraded in 0.01 M HCl at room temperature for 30 min. (b) Sample degraded in 1 M NaOH refluxed at 80 °C for 4 h. (c) Sample subjected to oxidative degradation. (d) Sample subjected to photo degradation.

separation of degradation products was achieved using a C_{18} column. The obtained m/z values in positive ESI mode were compared to the molecular weights of the degradation products. The fragmentation pattern was also investigated. Based on the molecular weight and the fragmentation pattern, the presence of degradation products was confirmed and also, structures could be proposed. The degradation pathway was outlined based on the results.

3. Results and discussion

3.1. Stability-indicating property

3.1.1. Hydrolysis

The rate of degradation in acid was faster as compared to that of alkali. The drug was found to be highly labile to acidic degradation. The reaction in 0.1 M hydrochloric acid at room temperature was so fast that around 60% of the drug was degraded in 30 min. Subsequently, studies were performed by reducing the molarity of hydrochloric acid to 0.01 M. Drug showed degradation around 10% within 30 min at room temperature associated with rise in a major degradation product at retention time 7.6 min in HPLC (Fig. 2(a)). Complete degradation of the drug was observed in 4 h when exposed to 0.01 M hydrochloric acid at room temperature.

Initially 0.1 M sodium hydroxide was refluxed at 80 °C for 8 h but no degradation was observed hence the strength of base was increased, 10–20% degradation was observed by refluxing drug solution with 1 M sodium hydroxide at 80 °C for 4 h forming degradation product at retention time 7.6 min in HPLC (Fig. 2(b)).

In both acid as well as base hydrolysis, the degradation products formed were same, which was confirmed by the study of mass values observed in mass spectra (Fig. 3(a) and (b)). The major degradation products showed molecular weight of m/z 298 in both acid and base hydrolysis. Also similar retention time as well as retardation factor was observed by HPLC and HPTLC for major degradation product formed under acid and base hydrolysis. LC–MS analyses of acid and base degraded sample showed peak for major degradation product at 5.49 and 5.42 min, respectively.

Mechanism for acid and base hydrolysis of tenatoprazole: It is well known that omeprazole undergoes hydrolysis and oxidation to give their corresponding degradation product [12]. The drug tenatoprazole upon degradation study followed the similar pathway of omeprazole due to there structural similarities The route of decomposition of tenatoprazole under acid and base hydrolysis is outlined in following scheme (Fig. 4).

The decomposition route is explained on the basis that under acidic conditions, the pyridinyl nitrogen is protonated to form pyridinium. Then the lone pair of electrons on the pyridinyl nitrogen

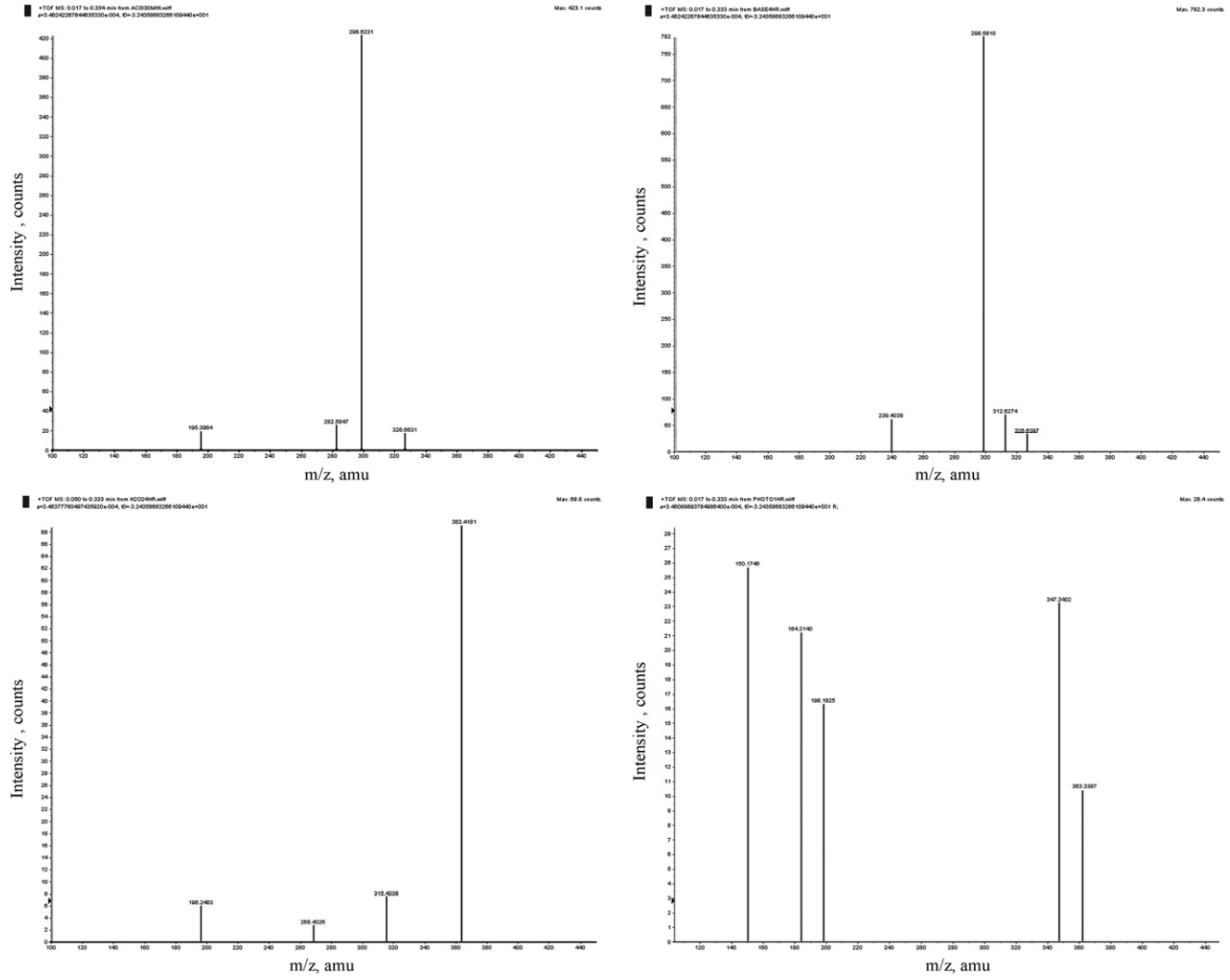

Fig. 3. Representative positive ESI-Quadrapole (+Q1) mass spectra of tenatoprazole. (a) Acid degradation product. (b) Base degradation product. (c) Oxidative degradation product. (d) Photo degradation product.

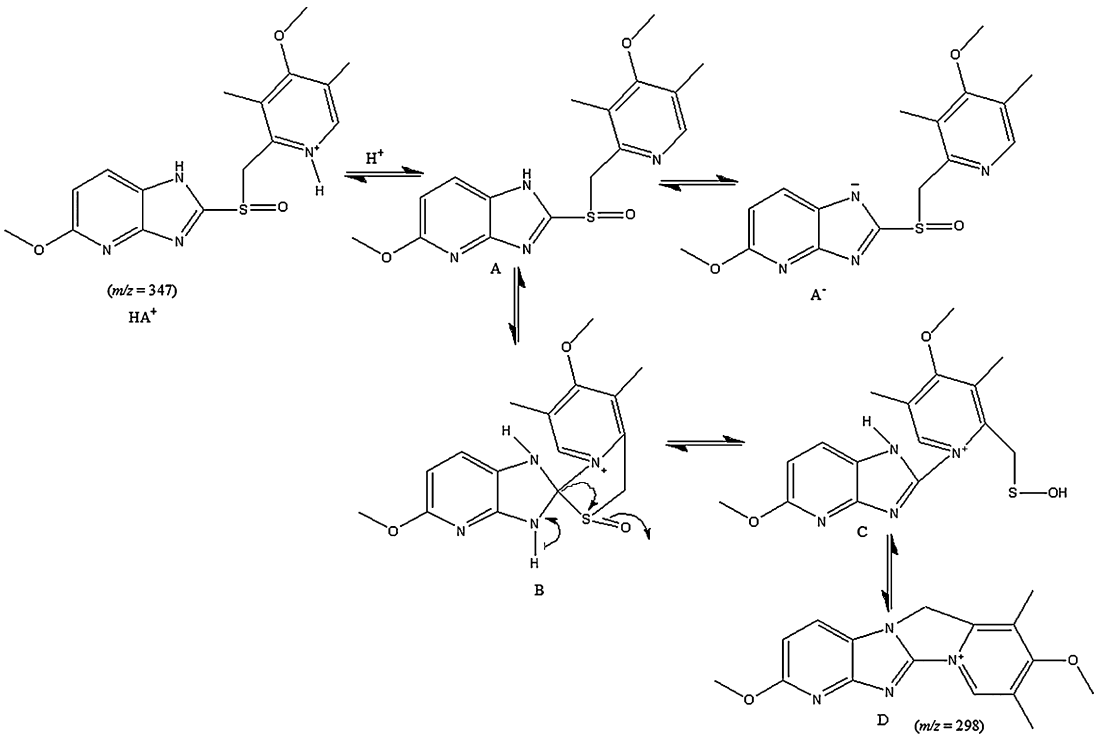

Fig. 4. Mechanism for acid and base hydrolysis of tenatoprazole.

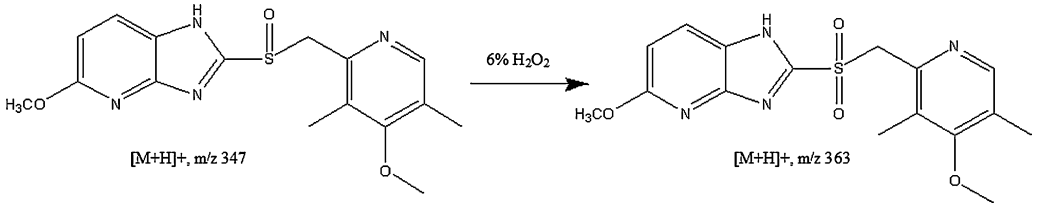

Fig. 5. Pathway for oxidation of tenatoprazole.

undergoes nucleophilic attack at the electron deficient carbon (2nd carbon) of the imidazole ring. This results in the formation of indene derivative of tenatoprazole. This is supported by the spectral data (Fig. 3(a)).

3.1.2. Oxidation

The drug was found to be unstable to oxidative degradation. The reaction in 30% H_2O_2 at room temperature was so fast that around 60% of the drug was degraded in 1 h. 8–10% degradation was observed when exposure to 6% H_2O_2 for 30 min forming major degradation product at 4.1 min. Around 20–25% degradation was observed when exposed to 6% H_2O_2 for 1 h forming degradation product at 4.1 min (Fig. 2(c)).

LC–MS analyses of degraded sample (6% H_2O_2 at room temperature for 30 min) were carried out; it indicated a peak at 4.1 min. The study of mass values indicated that degradation product at 4.1 min

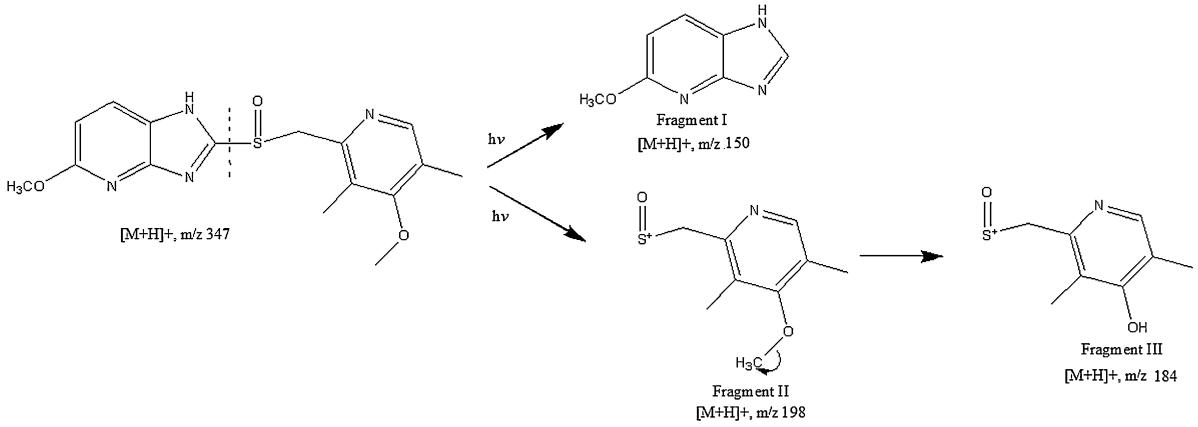

Fig. 6. Pathway for photo-degradation of tenatoprazole.

Table 1
Precision studies.

Concentration (μg/mL)	Measured concentration ± SD (μg/mL), RSD (%)	
	Repeatability ($n = 6$)	Intermediate precision ($n = 6$)
0.5	0.501 ± 0.00781, 1.558	0.4973 ± 0.00602, 1.212
2.5	2.477 ± 0.0451, 1.8238	2.4806 ± 0.02025, 0.8165
5.0	4.9486 ± 0.0463, 0.9357	4.937 ± 0.04613, 0.93437

to have molecular weight of m/z 363.41 (Fig. 3(c)). During H_2O_2 oxidation the sulphoxide gets converted to sulphonyl group which is supported by an increase in molecular weight by 16, and it is proved by spectral data (Fig. 5).

3.1.3. Photochemical degradation

Tenatoprazole was found to be unstable to photochemical degradation as more than 20% degradation was seen after exposing drug to sunlight for 1 h forming three major degradation products at 2.5, 5.5 and 6.2 min, respectively (Fig. 2(d)).

When tenatoprazole is exposed to sunlight; breaks sulpho bond into two fragments (fragment I m/z 198 and fragment II m/z 150). Fragment I further undergoes breakdown; methyl group is removed from 3-methoxy group of pyridine and results in the formation of 3-hydroxy pyridine with m/z 184 (fragment III). Possible degradation products formed under photochemical degradation are shown in following scheme (Fig. 6).

3.1.4. Dry and wet heat degradation

There was no significant degradation of solid tenatoprazole on exposure to dry heat at 50 °C for 2 months, which indicated that drug was stable against thermal stress. However, the exposure of drug to 40 °C/75% RH for 3 months resulted in slight degradation (less than 5%).

3.2. Validation of the stability-indicating method

The results of validation studies on the stability-indicating method developed for tenatoprazole in the current study involving methanol: 0.01 M acetate buffer pH 4.5 adjusted with glacial acetic acid (55:45) as a mobile phase are given below.

3.2.1. Linearity

The response for the drug was linear (0.999) in the concentration range between 0.1 and 10 μg/mL. The mean (±RSD) values of slope, intercept and correlation coefficient were 39435 (±1.30), 88371 (±1.57) and 0.999 (±0.876), respectively.

3.2.2. Precision

The results of the repeatability and intermediate precision experiments are shown in Table 1. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were <2%, respectively, as recommended by ICH guideline. Separation of the drug and different degradation products in stressed samples was found to be similar when analysis was performed on different chromatographic system on different days.

Table 2
Robustness testing[a] ($n = 3$).

Chromatographic changes				
Factor[b]	Level	t_R[c]	k[d]	T[e]
(A) Flow rate (mL/min)				
0.9	−1	15.00	4.00	1.10
1.0	0	14.12	3.70	1.08
1.1	+1	13.41	3.47	1.19
(B) % of methanol in the mobile phase (v/v)				
54	−1	15.12	4.04	1.17
55	0	14.12	3.70	1.09
56	+1	13.88	3.62	1.13
(C) pH of mobile phase				
3.7	−1	13.86	3.62	1.15
3.8	0	14.12	3.70	1.10
3.9	+1	14.88	3.96	1.22

[a] Average three concentrations 0.5, 2.5, 5 μg/mL.
[b] Four factors were slightly changed at three levels (1, 0, −1); each time a factor was changed from level (0) the other factors remained at level (0).
[c] Retention time.
[d] Retention factor.
[e] Tailing factor.

3.2.3. LOD and LOQ

The signal: noise ratios of 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 0.05 and 0.10 μg/mL, respectively.

3.2.4. Robustness of the method

Each factor selected (except columns from different manufacturers and solvents of different lots) was changed at three levels (−1, 0 and 1). One factor at the time was changed to estimate the effect. Thus, replicate injections ($n = 6$) of mixed standard solution at three concentration levels were performed under small changes of three chromatographic parameters (factors). Insignificant differences in peak areas and less variability in retention time were observed (Table 2). The resolution of drug in the mixture of stressed sample was found to be similar when studies were performed on different chromatographic system on different days indicating that the method has sufficient ruggedness.

3.2.5. Specificity

The specificity of the HPLC method is illustrated in Fig. 3 where complete separation of tenatoprazole in presence of its degradation products was noticed. The peaks obtained were sharp and have clear baseline separation. The resolution factor for drug from nearest resolving was >3 (Fig. 2). The photodiode array detector scanned all the components present in mixture in whole wavelength range from 200 to 400 nm and it indicated that there is no degradation peak (hiding) under or unresolved from the analyte peak (pure drug), which also reflected the specificity of the method.

3.2.6. Recovery studies

As shown from the data in Table 3 good recoveries of the drug in the range from 96% to 98% were made at various added concentrations, despite the fact that the drug was fortified to a mixture that contained drug as well as degradation product formed at various reaction conditions. Also, when the proposed methods were used for extraction and subsequent estimation of tenatoprazole

Table 3
Recovery studies ($n = 6$).

Drug	Label claim (mg per tablet)	Amount added (%)	Total amount (mg)	Amount recovered (mg) ± RSD (%)	Recovery (%)
Tenatoprazole	20	80	36	35. 8 ± 1.72	99.4
		100	40	38.9 ± 1.46	97.2
		120	44	43.2 ± 1.84	98.1

Table 4
Analysis of formulation.

Tenatoprazole (20 mg) enteric coated tablet	Tenatoprazole found (mg per tablet)	
	Mean ± SD ($n = 6$)	Recovery (%)
1st Lot (TNZ 0307)	19.84 ± 1.02	99.20
2nd Lot (TNZ 1007)	19.21 ± 0.08	96.05

from pharmaceutical dosage form after spiking with additional drug afforded recovery of 99%.

3.3. Analysis of marketed formulation

Two different lots of commercially available tenatoprazole tablet were analyzed using the proposed procedures and the results are summarized in Table 4.

4. Conclusion

Stability-indicating HPLC method was developed for tenatoprazole and validated as per ICH guidelines. UV detection allowed an accurate quantitation of chromophoric compounds, while MS detection was used to find the mass values of degradation products. In this study, intrinsic stability of tenatoprazole was established using various ICH recommended stress conditions. The drug as such was very stable in solid form and in methanolic solution. In the latter case, unknown decomposition products were formed under stress conditions. The drug was found to degrade extensively in acidic, oxidative and photolytic condition than in alkaline condition. Peak purity testing of degradation products were done by LC–MS. The method was validated for parameters like linearity, precision, accuracy, specificity, ruggedness, etc. and was also applied to real marketed samples. Thus, the method can be employed for analysis of drug during stability studies.

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