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Degradation Studies of Tadalafil: Identification, Isolation and Structure Characterization of Stress Degradation Product using LC-MS, Mass Mediated Prep-HPLC, NMR, HRMS

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The degradation behaviour of tadalafil performed under different stress conditions according to International Conference on Harmonization guidelines (ICH) was studied. A novel degradation product (DP) of tadalafil was observed only in acidic condition. An isolated degradation product [(*R*)-3-((1*H*-indol-3-yl)methyl)-4-(benzo[*d*][1,3]dioxole-5-carbonyl)-1-methylpiperazine-2,5-dione)] was identified by UPLC-MS technique and its structure was confirmed by high resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) studies. Acquity BEH C18 (50 mm × 2.1 mm, 1.7 µm) column was used in LC-MS, gradient run was performed with mobile phases 0.05% formic acid in acetonitrile and 0.05% formic acid in water, flow rate was 0.6 mL/min. The sample was diluted with acetonitrile and water.

Keywords: Tadalafil, Degradation product, LC-MS.

INTRODUCTION

Impotency is caused due to erectile dysfunction and this condition occurs in males. High blood pressure, cardiovascular disease, diabetes, stress, anxiety, age factor in men, obesity and high cholesterol are the various factors for causing erectile dysfunction. Tadalafil ((6R,12aR)-6-(benzo[*d*][1,3]dioxol-5-yl)-2-methyl-2,3,6,7,12,12a-hexahydropyrazino[1′,2′:1,6]-pyrido[3,4-*b*]indole-1,4-dione) can able to treat erectile dysfunction, benign prostatic hyperplasia and pulmonary arterial hypertension [1,2]. Tadalafil can acts as a PDE5 inhibitor and enhances the erectile function with the help of cGMP (cyclic guanosine monophosphate). With the intake of tadalafil pulmonary artery pressure can be decreased by widen the blood vessels in the lungs. Tadalafil has a longer half-life *i.e.*, 17.5 h [3] than other PDE5 inhibitors, other inhibitors like sildenafil, vardenafil having a half-life of 4-5 h [4]. Tadalafil works on the body within 0.5 h and functions up to 36 h. Few common side effects of tadalafil includes headache, stomach discomfort, burping, indigestion, acid reflux, back pain, muscle aches, flushing, stuffy and/or runny nose, however, in some of the serious side effects are prolonged erection and can lead to damage to the penis, problem on vision and loss of hearing [1]. Doctors do not recommend the people for taking nitrovasodilators and may result in a serious drop in blood pressure. Tadalafil is a generic medicine in United States and United Kingdom [5]. Some data found that 5 mg of tadalafil can lower the urinary tract symptoms and this had a lower adverse effects [6]. United States approved tadalafil for treating pulmonary arterial hypertension in 2009 [7].

Most of the API's are prone to be unstable under various conditions, which results in the loss of activity of the drug or may lead to adverse effects. With the help of the forced degradation studies one can know how to overcome the loss of the drug activity. Forced degradation of the drug was carried out in various stress conditions as recommended by the ICH guidelines [8-10]. Literature survey reveals that some of the analytical methods are available for the drug, which are stress degradation studies on tadalafil and development of a validated stability-indicating LC assay for bulk drug and pharmaceutical dosage form [11], pharmaceutical formulations [12] and *in vitro* dissolution samples [13,14].

The present study is focused on the forced degradation studies of tadalafil under different conditions. The degradation product was characterized by HRMS and NMR.

EXPERIMENTAL

Tadalafil was received as a gift sample from a reputed manufacturing unit in Hyderabad, India. High grade pure buffers and HPLC grade solvents *viz*. trifluoroacetic acid, hydrochloric acid, hydrogen peroxide, sodium hydroxide, acetonitrile and methanol were purchased from Merck, India. Optima LC/MS grade acetonitrile, methanol and formic acid were procured from Fisher Scientific Chemicals. The purified water utilized during analysis was obtained from Milli-Q plus purification system (Millipore, Amsterdam, Netherlands).

Ultra performance liquid chromatography-mass (UPLC-MS) spectrometry: Waters Acquity UPLC with photodiode array detector (PDA) was used for liquid chromatography (LC) separation. Acquity BEH C18, 2.1 mm × 50 mm, 1.7 µ column was used for analysis and obtained from Waters India limited, Mobile phase consisting of A: 0.05% formic acid (aq.), B: 0.05% acetonitrile with linear gradient was used. The gradient started from 3% (B) to 98% (B) in 2.5 min and then hold at 98% (B) till 3.4 min and then stabilized to 3% (B) till 4.0 min. Acetonitrile:water (80:20) was used as diluent for sample preparation. The data was monitored at maxplot for all the components present in the analysis.

Waters SQD-2 single quadrupole mass spectrometer operated in dual polarity-positive and negative with electrospray ionization source (ESI) were used. Mass range was 100-1500 Daltons (Da) in full scan mode. Source parameters include capillary voltage and source temperature were set at 3.5 kV and 120 ºC, respectively desolvation temperature was 350 ºC, desolvation gas flow was 750 L/h and cone gas flow was 50 L/h. Masslynx software controlled both the LC and MS instrument.

High resolution mass spectrometry: Thermo Q Exactive orbitrap MS was used to run HRMS analysis. ESI ion source; source parameters were spray voltage 3.5 kV, Aux gas heater temperature 400 ºC, capillary temperature 300 ºC, sheath gas flow rate 53 arb, aux gas flow rate 14 arb and sweep gas flow rate 3 arb. Reserpine was used to check the accuracy of the mass instrument and the monoisotopic mass $[M+H]^+$ of reserpine was 609.2807 Da. Dionex ultimate 3000 LC was used. Data was acquired in both positive and negative modes. The Xcalibur software was used to control the instrument.

Mass mediated preparative HPLC: Waters mass mediated preparative HPLC with 2545 pump, PDA 2998 detector, Acquity QDA mass detector and 2767 sample manager were used. Masslynx data handling software was used to operate the instrument. Waters X-Bridge C18 column with dimensions 250 mm × 19 mm, 5 µm was used to separate the degradation product. In QDA detector mass capillary voltage was maintained at 1.5 kV, source temperature 150 ºC and probe temperature at 600 °C, cone voltage 20 V. The desolvation gas flow was set at 650 L/h. Cone gas flow was 50 L/h. 20 mM Ammonium acetate (aq.) and acetonitrile (30:70, v/v) was used as a makeup solvent with makeup flow of 0.3 mL/min to the mass detector and 1:1000 splitting ratio was maintained for the proper ionization.

NMR spectroscopy: NMR analysis of tadalafil (API) and acid degradation product were taken on Agilent MR400MHz. ONE NMR probe was connected to NMR with Z-gradient shim. A 298 K probe temperature was applied with fine tuning. Tetra methyl silane (TMS) set at 0 ppm in ^{1}H NMR and referenced DMSO-d_6 septet at 39.5 ppm in carbon NMR.

Two-dimensional (2D) analysis: Correlation between proton-proton has been performed under homonuclear gDQ-COSY. 1J correlation between proton-carbon was performed under gHSQC experiment. The gHMBC has been performed to reveal the exact structure of degradation product.

Sample preparation

For forced degradation: Various stress parameters *i.e.* acidic, basic, oxidative, thermal and photolytic conditions were followed as per ICH guidelines [10].

Acid degradation: Tadalafil (300 mg) was mixed in a 5 mL of 1 N HCl and refluxed at 60 ºC for 10 h. The aliquots were taken for further analysis.

Base degradation: Tadalafil (300 mg) was mixed in a 5 mL of 1 N NaOH and refluxed at 60 ºC for 10 h. The aliquots were taken for further analysis.

Oxidative degradation: Tadalafil (300 mg) was mixed in a 5 mL of 3% H_2O_2 and the mixture was kept at room temperature for 48 h to record for further analysis.

Photolytic degradation: Photolytic degradation was carried out under dual wavelengths, short wave UV light at 254 nm and another in long wave UV light at 366 nm (CAMAG UV Cabinet). Around 300 mg of tadalafil was kept in a clean petri dish under long and short UV light for a period of 48 h, no physical change was observed and the sample was taken for further analysis.

Thermal degradation: Tadalafil (300 mg) was kept at 120 ºC for 48 h in hot air oven, no physical change was observed and the sample was taken for further analysis.

For NMR analysis: Tadalafil (10 mg) was dissolved in deuterated DMSO-d_6 solvent and analyzed.

Preparation of degradation sample for purification: Degradation was observed only in acid condition. Acid degraded sample was neutralized with saturated solution of NaOH (aq.) and the resultant solution was lyophilized to get crude solid sample. The sample was then dissolved in 4-5 mL of mobile phase for further purification.

Degradation behaviour of tadalafil: No degradative products were found in basic, oxidative, thermal and photolytic conditions, which confirms the stability of tadalafil API. The drug was found to be labile to acid hydrolysis, as a result of 12.78% degradation was found (1 N HCl reflux at 60 ºC, up to 10 h) with 9.72% of degradation product. Table-1 shows the degradation details.

RESULTS AND DISCUSSION

The degradant was formed after specific time interval (Fig. 1). The solutions of all the stress study samples were analyzed individually in mass spectrometer. One significant degradation

TABLE-1
FORCE DEGRADATION STUDY DETAILS OF TADALAFIL

Conditions	Degradation product (%)	API (%)
Tadalafil API	–	99.90
Acid (1N HCl)	9.72	87.22
Base (1N NaOH)	–	99.55
Oxidation (3% H_2O_2)	–	98.80
Thermal (120 °C for 48 h)	–	99.65
Photolytic (254 and 366 nm for 48 h)	–	99.73

product was formed, identified, isolated and characterized by LCMS, HRMS, NMR (1D and 2D).

Isolation of degradation product of tadalafil: The degradation was observed in acid stress condition with adequate percentage formation of 9.72% (Table-1). Purification was carried out by using 0.1% trifluoroacetic acid (aq.) and acetonitrile as a mobile phase with X-Bridge C18 (250 × 19 mm); 5 µ column. Acid degraded sample was diluted with mobile phase and the sample was purified by using mass mediated Prep HPLC.

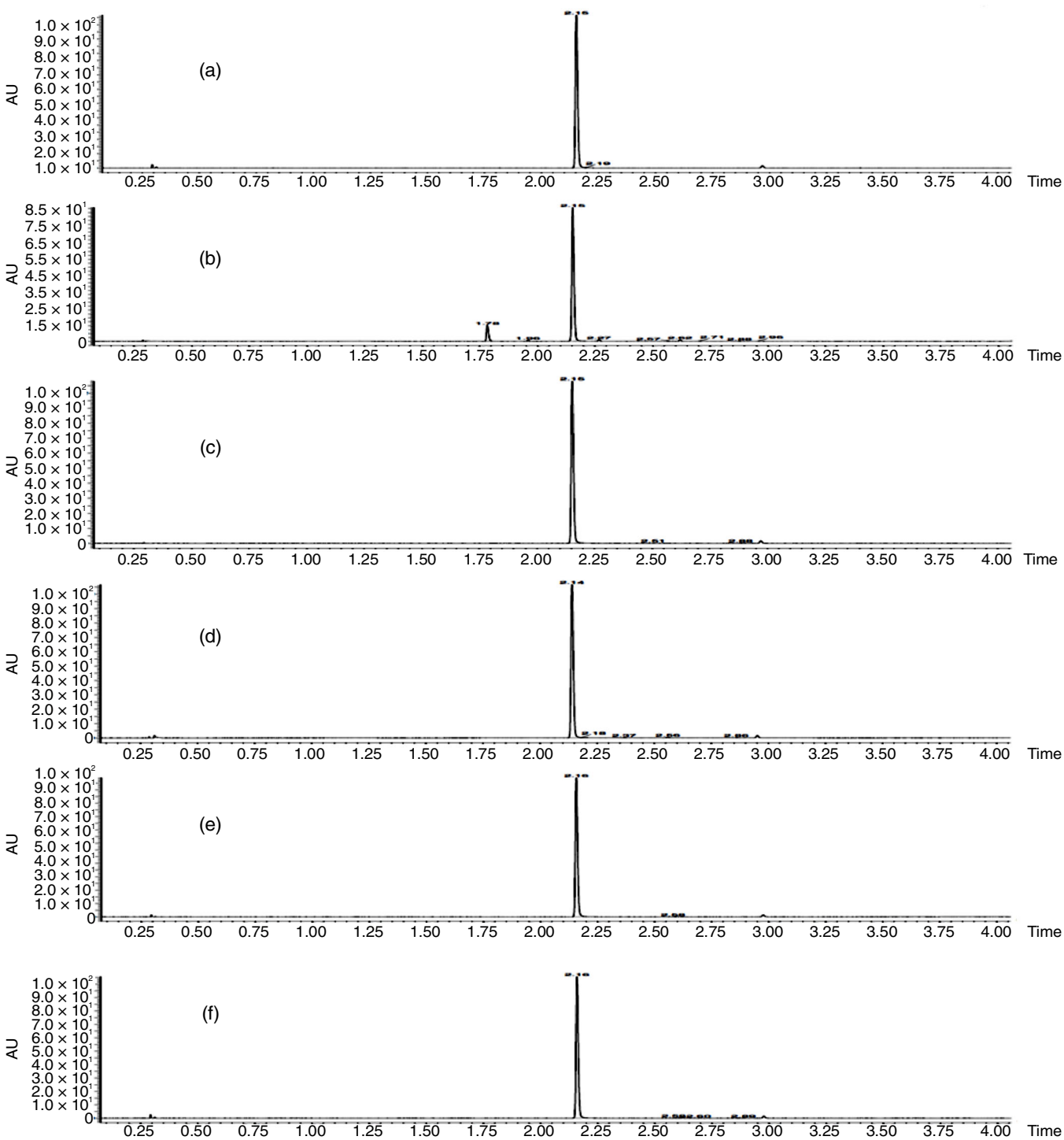

Fig. 1. Tadalafil and its stress degradation chromatograms (a) tadalafil (API), (b) acid degradation, (c) base degradation, (d) oxidative degradation, (e) thermal degradation, and (f) photolytic degradation

Then the sample solution was injected in consecutive injections and the fractions had been collected on the basis of molecular ion peak *i.e.* 406.15 (M+H), fraction was lyophilized to get free solid.

Structure confirmation of degradation product

Characterization of tadalafil (API): Tadalafil (API) has been recorded 1D & 2D NMR experiments and the data are shown in Table-2. Proton NMR of API shows 7 aromatic protons around 7-8 ppm, one indole –NH proton at 11.02 ppm and $–CH_2$ protons flanked between two oxygen atoms was appeared at 5.92 ppm. The indole ring attached chiral proton was observed at 6.13 ppm as broad peak, which has been confirmed further by VT NMR. Other two $–CH_2$ groups and one $–CH_3$ were seen at around 2.9 to 4.7 region and other chiral proton triplet was seen at 5.5 ppm.

Characterization of degradation product: HRMS was used as a preliminary step in impurity characterization to obtain the exact mass of the compound. The protonated exact mass $[M+H]^+$ of degradation product was 406.1395 (0.52 ppm) for the calculated molecular formula of $C_{22}H_{19}N_3O_5$. The degradation product has 16 mass units (daltons) excess as compare to the API compound (Fig. 2).

Precise structure of degradation product has been confirmed with the help of ^1H, ^{13}C NMR and 2D experimental analysis. Based on ^1H NMR data, it is understood that the compound is rotameric in nature and in ^{13}C NMR, one extra carbonyl carbon was observed at 162.98 & 163.37 ppm (Fig. 3), as the compound has *tertiary* amide bond and existed as rotamers. In ^1H NMR, 8 aromatic and one indole –NH at 12 ppm and $–CH_2$ protons flanked between two oxygen atoms at 6.2 ppm and other two $–CH_2$ groups and one $–CH_3$ around 2.9 to 4.7 ppm and other chiral proton triplet was obtained at 5.5 ppm. In ^1H-^{13}C HMBC experiment, observed key correlation of H22, H26 protons showing ^3J connectivity to C10 carbon. Fig. 4 shows the gHMBC spectrum of degradation product with key correlation and all the above information suggesting that six-membered ring got opened at 2nd position of indole nitrogen and the chiral centre carbon was oxidized. ^1H & ^{13}C chemical shift values has been tabulated in Table-3.

MS/MS analysis of degradation product: In MS/MS analysis, fragmentation obtained by applying collision energy (CE), with linear increase in CE, better fragmentation pattern for degradation product (406 amu) was found at 30 CE. A

TABLE-2
ASSIGNMENTS OF ^1H AND ^{13}C NMR SIGNALS OF TADALAFIL API

Atom No.	Type of atom	^1H Chemical shift (ppm); Coupling const (*J*)	^{13}C Chemical shift (ppm)
1	CH	6.99 (t, 8.0 Hz, 1H)	118.84
2	CH	7.06 (t, 7.2 Hz, 1H)	121.21
3	CH	7.28 (d, 8.0 Hz, 1H)	111.28
4	C	–	136.17
5	C	–	125.73
6	CH	7.53 (d, 7.6 Hz, 1H)	118.08
7	NH	11.02 (s, 1H)	-
8	C	–	133.91
9	C	–	104.72
10	CH	6.13 (s, 1H)	55.25
11	N	–	–
12	CH	4.38 (dd, 11.6 Hz, 4.4 Hz, 1H)	55.48
13	CH_2	2.97 (dd, 15.2 Hz, 12.0 Hz, 1H), 3.51 (dd, 16.0 Hz, 4.4 Hz, 1H)	23.1
14	C	-	166.86
15	CH_2	3.92, 4.15 (d, 17.2 Hz, 2H)	51.44
16	N	–	–
17	C	–	166.54
18	O	–	–
19	O	–	–
20	CH_3	2.93 (s, 1H)	32.85
21	C	–	136.96
22	CH	6.86 (s, 1H)	106.94
23	C	–	147.01
24	C	–	146.03
25	CH	6.78 (d, 8.0 Hz, 1H)	107.96
26	CH	6.78 (d, 8.0 Hz, 1H)	119.28
27	O	–	–
28	CH_2	5.92 (s, 2H)	100.87
29	O	–	–

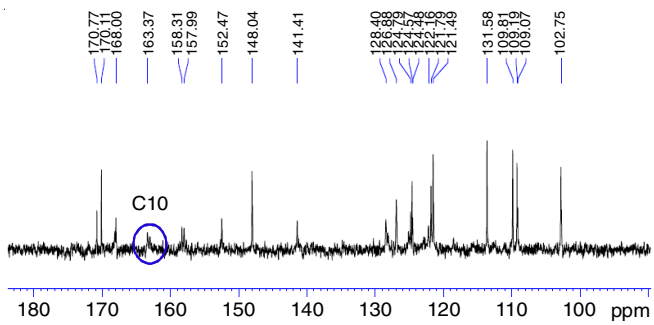

Fig. 3. ^{13}C spectrum of degradation product

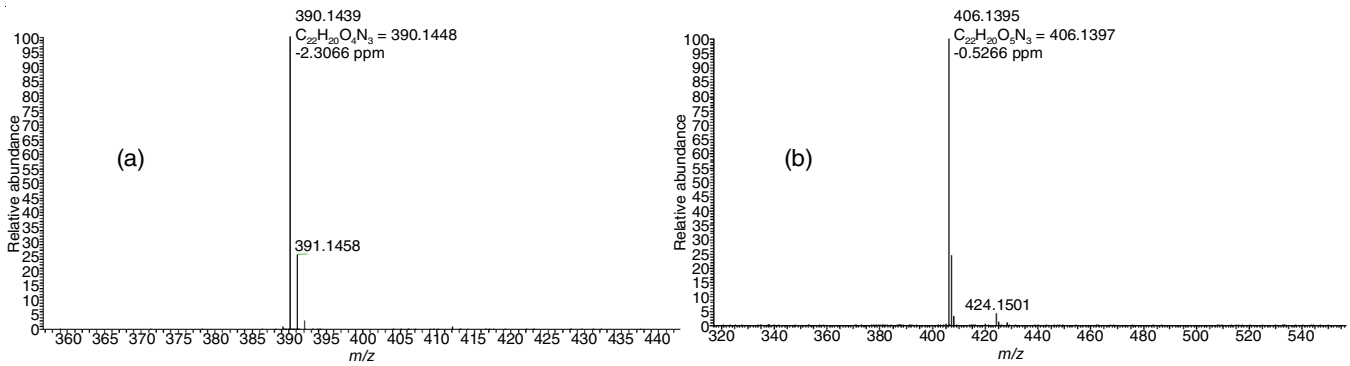

Fig. 2. HRMS spectrum of (a) tadalafil and (b) degradation product

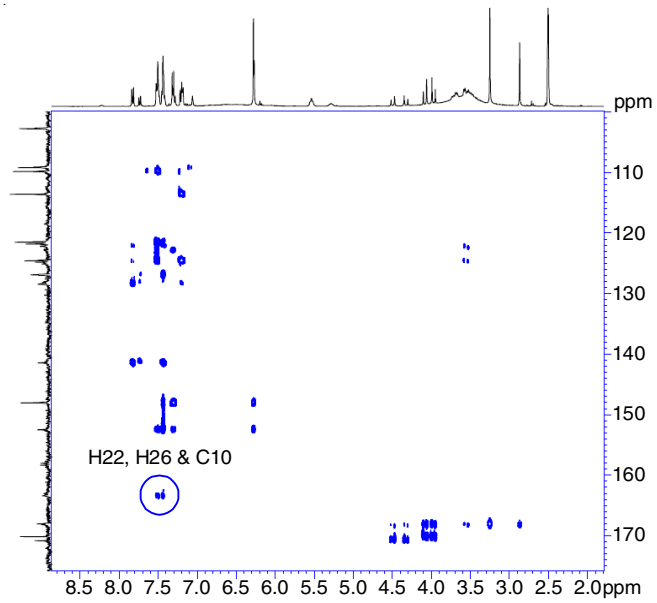

Fig. 4. ^{1}H-^{13}C gHMBC spectrum of degradation product with key correlation

prominent mass peak is 305 amu and other masses are 124 amu, 130 amu, 215 amu, 289 amu, 335 amu and 378 amu. MS/MS spectrum of degradation product is shown in Fig. 5.

Conclusion

In this work, the identification, isolation and the structure characterization of stress degradation product of tadalafil was performed.Tadalafil was found to be stable under stressed basic, oxidative, thermal and photolytic conditions but labile to acid degradation. Only one novel degradation product was isolated, identified and characterized. The degradation product was formed by six membered ring ruptured at the 2nd position of indole nitrogen and the chiral center carbon was oxidized.

TABLE-3
ASSIGNMENTS OF ^{1}H AND ^{13}C NMR SIGNALS OF DEGRADATION PRODUCT OF TADALAFIL

Atom No.	Type of atom	^{1}H Chemical shift (ppm); Coupling const (*J*)	^{13}C Chemical shift (ppm)
1	CH	7.19 (t, 7.2 Hz, 1H)	121.49
2	CH	7.41 (m, 1H)	128.4
3	CH	7.51 (m, 1H)	113.58
4	C	–	141.41
5	C	–	124.48&124.57
6	CH	7.72, 7.81 (d, 8.0 Hz, 1H)	121.49&121.79
7	NH	12.02 (s, 1H)	–
8	CH	Broaden	Broaden
9	C	–	122.16
10	C	–	162.98&163.37
11	N	–	–
12	CH	5.28, 5.53 (t, 6.4 Hz, 8.2 Hz, 1H)	52.99
13	CH_2	3.53, 3.68 (dd, 17.6 Hz, 8 Hz, 2H)	22.15&22.63
14	C	–	170.11&170.77
15	CH_2	3.94, 4.05, 4.30, 4.46 (d, 18.8 Hz, 2H)	49.64&50.97
16	N	–	–
17	C	–	168&168.14
18	O	–	–
19	O	–	–
20	CH_3	2.86&3.24 (s, 3H)	35&36.25
21	C	–	122.85
22	CH	7.43 (d, 2 Hz, 1H)	109.81
23	C	–	148.04
24	C	–	152.47
25	CH	7.30 (d, 8.0 Hz, 1H)	109.07&109.19
26	CH	7.49 (m, 1H)	126.88
27	O	–	–
28	CH_2	6.26&6.27 (s, 2H)	102.75
29	O	–	–
30	O	–	–

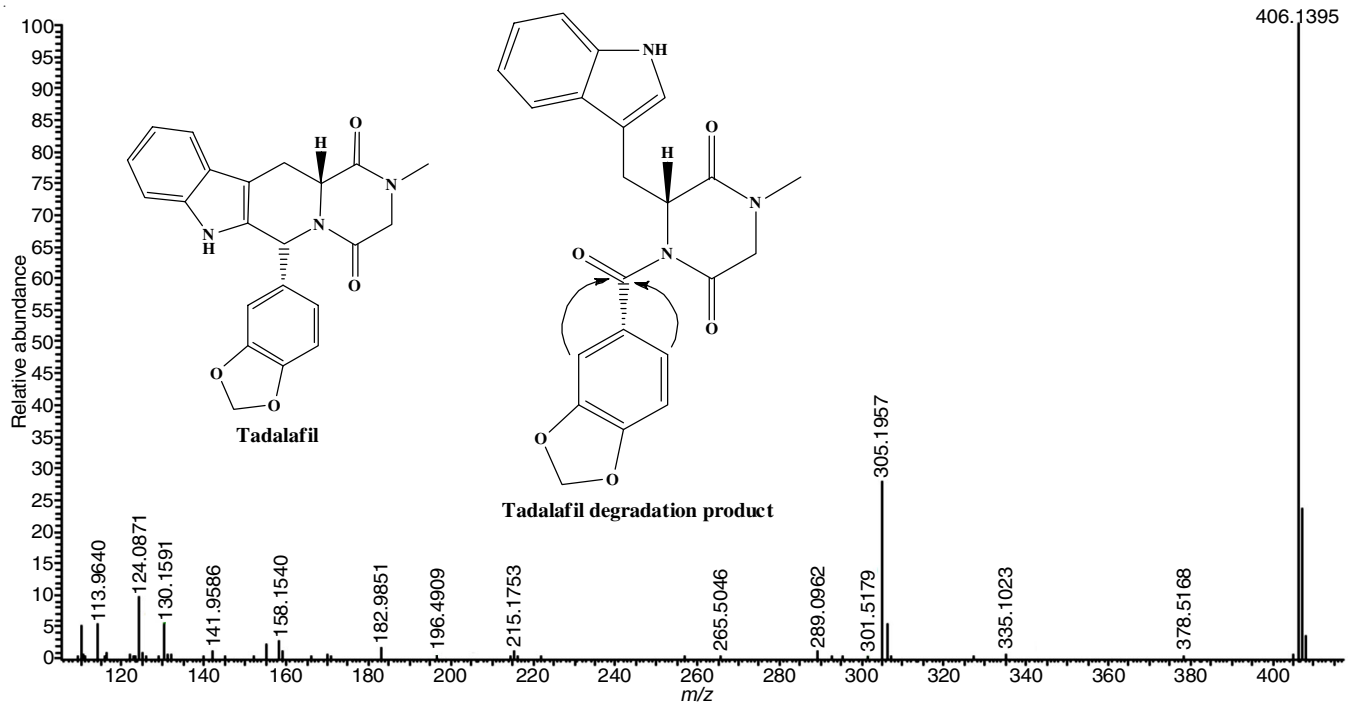

Fig. 5. MS/MS spectrum of degradation product

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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