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Application of UHPLC method for separation and characterization of major photolytic degradation products of trazodone by LC-MS and NMR

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Abstract

Drug degradation products are type of drug impurities which affects the safety of the drug products. Trazodone (TZD) is an antidepressant drug subjected to forced degradation as per ICH embedded guidelines for predicting the drug degradation products. It undergoes degradation in acidic hydrolysis, peroxide induced oxidation and upon exposure to day light and results in formation of ten degradation products (DPs). A UHPLC method was developed to separate TZD and its DPs using the stationary phase of Acquity UPLC CSH C_{18} column (100 x 2.1 mm, 1.7 µm) and mobile phase of solvent-A (10 mM ammonium acetate, pH 8.5) and solvent-B (Methanol) at a flow rate of 0.25 mL min^{-1} in gradient elution. This method was transferred to quadrupole time-of-flight tandem mass spectrometer (QTOF-MS/MS) for identification of DPs. Very interestingly, four dimer DPs were found in photolytic degradation condition and they are found to be isomers. The major isomer (DP-10) was isolated by using preparative HPLC and its structure was identified using proton and carbon NMR. Three N-oxide DPs were also observed and the site of N-oxidation was identified by using atmospheric pressure chemical ionization mass spectrometry (APCI-MS). The developed UHPLC method was validated as described in ICH prescribed guideline and USP general chapter on method validation. The proposed validated stability indicating assay method can be used for identification and quantification of TZD and its DPs in the quality control lab for product release testing and stability studies of the drug in much lesser time with greater selectivity.

Keywords: Trazodone (TZD); forced degradation; UHPLC; LC-ESI-QTOF-MS/MS; NMR; Dimer degradation products.

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Introduction

Trazodone (TZD) is a triazolopyridinone derivative antidepressant drug belongs to the class of the serotonin antagonist (5HT-2) and reuptake inhibitors (SRIs).[1] TZD is chemically described as 2-(3-(4-(3-chlorophenyl)piperazin-1-yl)propyl)-[1,2,4]triazolo[4,3-a]pyridin-3(2H)-one hydrochloride. It is a multifunctional drug having dose dependent pharmacological activities, at a lower dose it worked as a hypnotic due to blockage of $5HT_{2A}$, H_1-histamines and α adrenergic receptors while at a higher dose it shows an antidepressant action because of serotonin antagonist and reuptake inhibitor (SARI) activity.[2] Different drug formulations of TZD are commercially available for the treatment of major depression, generalized anxiety disorder, uni-polar depression and insomnia.[3-4]

Forced degradation study is very important in the initial stage of pharmaceutical drug development process because it deals with the identification and characterization of possible degradation products (DPs) which can originate from the drug substance and/or drug product.[5-7] Hence, it is necessary to find-out the intrinsic stability of the drug as well as drug degradation pathways. The forced degradation studies are also useful for self life determination, designing of manufacturing process, formulation and packaging development. Thus, various global regulatory bodies like international conference on harmonization (ICH) guidelines emphasized to conduct the forced degradation and characterization of all the degradation products (DPs) in an early stage of drug discovery and development process because the presence of DPs may impose the risk of higher and unpredictable toxicity along with formulation related defects.[8-9] Modern analytical techniques like UHPLC-ESI-QTOF-MS/MS and NMR are very promising in impurity profiling areas to identify and characterize all the types of impurities.[10-14]

The literature shows stability indicating HPLC method [15-16] and bio-analytical methods for estimation of trazodone and/or with its metabolite in biological samples.[17-21] But no analytical

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method was found for identification and characterization of forced degradation products of TZD. Hence, the authors have developed a UHPLC method for separation of all the degradation products of the drug followed by characterization of their chemical structures using UHPLC-ESI-QTOF-MS/MS and NMR.

Experimental

Chemicals and Reagents

The drug was obtained as a gift sample from INTAS Pvt. Ltd. (Gujarat, India). Gradient grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck Specialities Pvt. Ltd. (Mumbai, India). HPLC Buffer grade-Ammonium acetate and formic acid; Analytical reagent grade-sodium hydroxide (NaOH), hydrochloric acid (HCl) and hydrogen peroxide (30% v/v) were purchased from fine chemicals Pvt. Ltd. (Ahmadabad, India). High purity water was obtained by Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

Instrumentation

The Acquity UPLC H-Class system from Waters (Waters, Milford, MA, USA) composed of a quaternary solvent manager, sample manager of flow-through needle design (SM-FTN), temperature controlled multiple column compartments and PDA detector was used for chromatographic method development. The separation system was controlled by Empower-3 software. LC-MS/MS characterization was carried out using an Agilent 1290 series LC instrument (Agilent Technologies, USA) attached to a quadrupole – time of flight mass analyzer (Q-TOF, Agilent 6540, Agilent Technology, USA) and coupled with electrospray ionization (ESI) and/or an atmospheric pressure chemical ionization (APCI) source. The mass spectrometric data analysis was carried out using Mass Hunter workstation software. NMR studies were performed using 500 MHz NMR (AVANCE III HD-500, Bruker, Switzerland) spectrometer. TMS (Trimethylsilane) was used as internal standard. A mixture of CDCl3

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with *d6*- DMSO and $CDCl_3$ were used as solvents for proton and carbon NMR, respectively. A photo stability chamber (model no. OPSH S/G-16, Osworld science Pvt Ltd India) consisting of UV and fluorescent lamps as described in ICH-Q1B guideline [21] was used for the photo-degradation study. Hydrolytic and thermal degradation studies were performed using high precision water bath (temperature control ± 1 ºC) and hot air oven (Oswarld scientific Pvt Ltd. India), respectively. Samples were dissolved using an ultra-sonicator from Power Sonic-405 (Hwashin Technology Co., Seoul, Korea). A pH meter from pH tutor (Eutech Instruments, Singapore) was used to measure pH of solution.

Optimized Chromatographic and Mass spectrometric Conditions

Optimum chromatographic separation of drug and DPs was achieved by using Acquity UPLC CSH C_{18} (100 x 2.1 mm, 1.7 µm) column with gradient mobile phase of solvent-A (10 mM ammonium acetate buffer, pH 8.5) and B (Methanol) at a flow rate of 0.25 mL min^{-1}. The gradient program was set as follows: (Time in min / % of solvent-A): 0-0.5/60, 0.50-4.0/30, 4.0-11.0/15, 11.0-11.50/60, 11.50-15.0/60. Obtained chromatograms were monitored at wavelength of 252 nm. The column oven and auto sampler temperatures were maintained at 25 °C and 10 °C respectively. The sample injection volume of 1 µL was used in study. For LC-ESI-QTOF-MS/MS analysis, the source condition for ESI was optimized as follows: the fragmentor voltage at 140 V, the capillary voltage at 3500 V and the skimmer voltage at 65 V. Nitrogen gas was used as the drying (325°C; 10 L/min) and nebulizing (40 psi) gas. In collision induced dissociation (CID) experiment, keeping MS1 static, the quadrupole mass analyzer was used for the precursor ion scanning and the TOF mass analyzer was used for the product ions scanning. All the mass spectra were recorded under identical experimental conditions and an average of 25 scans. In APCI ionization, source current and vaporizer temperature were set at 3 µA and 400 °C, respectively. All the remaining source parameters were maintained same as in ESI.

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Forced degradation study

Acid hydrolysis (3N HCl for 24 h), basic hydroxylation (3N NaOH for 24 h) and neutral hydrolysis (water for 48 h) were carried out by refluxing stressed solutions under heat at 70 °C. Oxidative degradation was carried out using 30% (w/v) hydrogen peroxide solution at room temperature for 24 h. In photolytic degradation, solid and solution state drug samples were kept in photo-stability chamber and irradiated with UV-light and visible light of not less than 200 Wh/m^2 and 1.2 million lux hr, respectively as per ICH guidelines.[22] A parallel set of the drug samples were stored in dark at same temperature to serve as a control. Thermal degradation of solid sample was performed by making 1 mm thickness layer of drug sample and kept it at 90 °C for 7 days.

Sample preparation

All stress degradation experiments were carried out on 1 mg mL^{-1} of TZD solutions. After obtaining sufficient degradation, 1 mL of stressed solution was taken and allowed to cool at room temperature followed by neutralization and/or dilution with diluent (ACN: water-40: 60 v/v) to get assay concentration of 100 ppm and sonicated for 5 min. The final injection solution was filtered through a 0.22 µm whatmann filter paper before injected into the chromatographic UHPLC system. All solutions were stored in an amber color flask at approximately 4 °C temperature.

Method validation

The chromatographic method was validated as per ICH guideline Q2 (R1) and USP general chapter on method validation for specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision and robustness. [23-24]

Preparative chromatographic conditions for isolation of DP-10

Waters (Waters, M.A, USA) preparative HPLC system equipped with 515 binary HPLC pumps and 2489 UV–visible detector was used for isolation of major degradation product

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(DP-10). An optimized chromatographic condition for isolation includes stationary phase of Waters Xbridge C18 column (250 × 19 mm, 5µm, 130 Å) and isocratic mobile phase of 10 mM ammonium acetate buffer (pH 6.5) and Methanol (60:40 % v/v) at a flow rate of 12 mL min^{-1}. The 80 % of DP-10 peak was collected after each injection in preparative system. The purity of isolated fraction was confirmed by re-injecting into UHPLC system and it was found to be more than 99 % pure. The isolated fraction was concentrated and dried using rotary evaporator at 45 °C temperature under vacuum. The corresponding preparative chromatogram is given in supplementary information (Fig. Sa).

Results and Discussion

Method Development and Optimization

The chromatographic conditions were optimized to attain well separation of drug and its DPs. Systematic trials on chromatographic separation conditions were based on improving peak shape and resolution between peaks of TZD and its DPs. Being a non polar drug, TZD showed better selectivity and retention on C18 column. In initial trials on different column chemistries suggested that Acquity UPLC CSH C18 (100 x 2.1 mm, 1.7 µm) column was appropriate for method development. Isocratic and gradient trails using MeOH/ACN as an organic phase and water as aqueous phase on above column shows insufficient separation of DP-7, DP-8, DP-9 and DP-10. Hence, mass compatible buffers like ammonium formate and ammonium acetate at pH ± 1.5 of drug pKa were tried. The gradient trials with MeOH/ACN and buffers at acidic pH (1-5) shown co-elution (DP-7, DP-8 and DP-9). While the trials with buffers at basic pH (8-11) shows improved separation as compared to acidic buffers. Finally well-resolved degradation product peaks with a sharp and symmetrical drug peak was obtained on stationary phase of Acquity UPLC CSH C_{18} (100 x 2.1 mm, 1.7 µm) column with a mobile phase containing gradient mixture of solvent-A (10 mM ammonium acetate buffer pH 8.5) and B (Methanol) as: (Time in min / % of solvent-A): 0-0.5/60, 0.50-4.0/30, 4.0-

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11.0/15, 11.0-11.50/60, 11.50-15.0/60. The flow rate was set at 0.25 mL min^{-1}. All the chromatograms were processed and analyzed at wavelength of 252 nm.

Degradation behavior of TZD

TZD was degraded in the presence of acid hydrolytic, oxidative and photolytic stress degradation conditions while it was found to be stable in remaining stress degradation conditions. A total of ten DPs were observed and overlaid chromatogram was given in Fig. 1. Minor degradation (2.8 %) of the drug was observed in acid hydrolysis to give rise of two DPs (DP-4 and DP-6). Three DPs (DP-1, DP-2 and DP-3) were obtained in peroxide stress condition with percentage of degradation of 5.73 %. A solid sample of the drug was remained stable in both UV and visible light while solution state sample was extensively degraded in UV-light (42.5 %) and five DPs were obtained (DP-5 and DP-7 to DP-10) while one degradation product (DP-10, 1.5%) was observed under visible light.

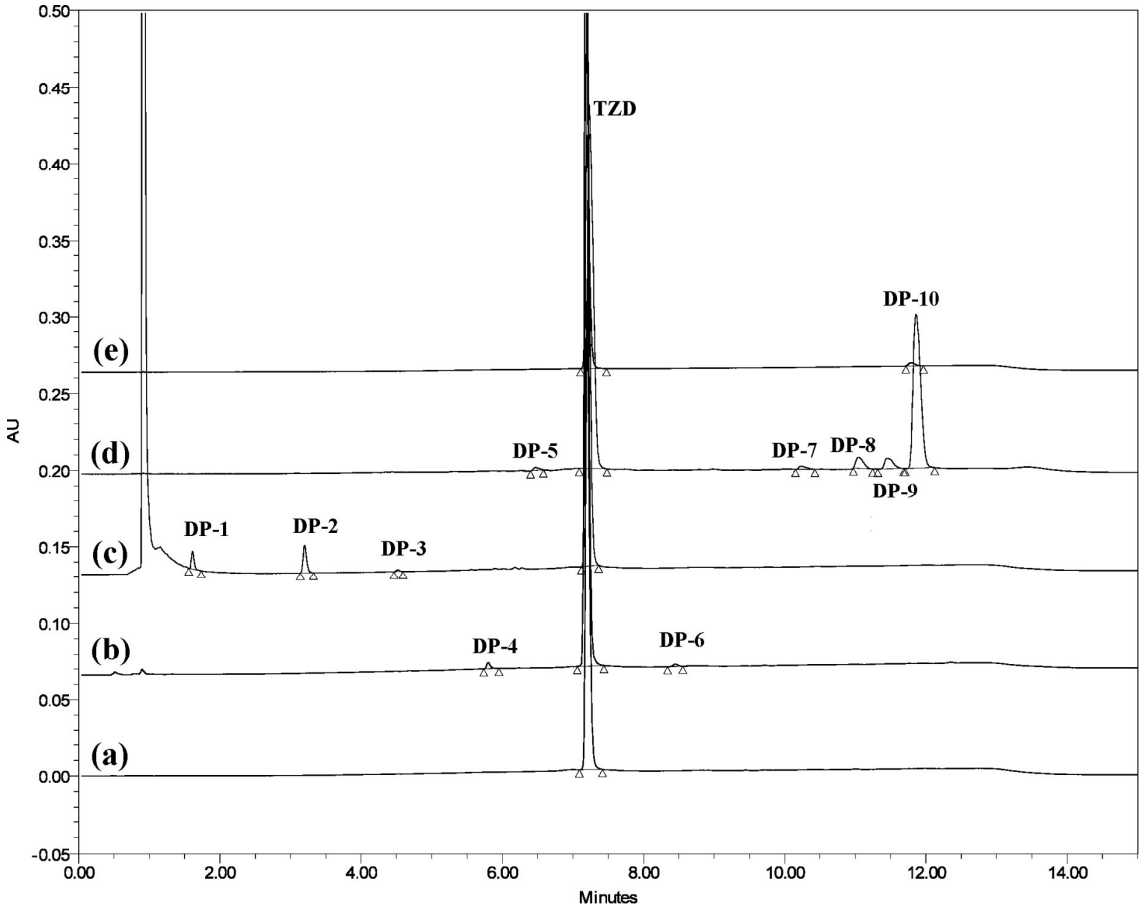

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Fig. 1. UHPLC-PDA Chromatograms of [a] TZD (100 ppm), [b] Acid hydrolysis (3N HCl, for 24 h), [c] Peroxide induced oxidation (30% w/v H_2O_2 for 24 h), [d] Solution sample exposed to UV light and [e] Solution sample under visible light.

LC–MS/MS and NMR of TZD and DPs:

LC-ESI-QTOF-MS/MS and NMR experiments were carried out to characterize the drug and its degradation products. Most possible structures were proposed based on *m/z* values of $[M+H]^+$ of precursor and product ions, fragmentation pattern of precursor ion and elemental composition derived from high resolution mass spectrometric (HR-MS) data. Molecular formulas corresponding to the observed *m/z* and to the calculated *m/z* with mass error in ppm are given in Table 1.

Table 1. high resolution mass data corresponding to elemental composition of TZD and its DPs

Description	Molecular Formula $[M+H]^+$	Observed *m/z*	Calculated *m/z*	Error (ppm)
TZD	**$C_{19}H_{23}ClN_5O^+$**	**372.1583**	**372.1586**	**0.81**
	$C_{13}H_{18}ClN_2^+$	237.1163	237.1153	-4.22
	$C_{11}H_{15}N_4O^+$	219.1239	219.124	0.46
	$C_{11}H_{14}ClN_2^+$	209.0844	209.084	-1.91
	$C_{10}H_{11}ClN_2$	194.0612	194.0605	-3.61
	$C_9H_{10}N_3O^+$	176.0820	176.0818	-1.14
	$C_8H_9ClN^+$	154.0429	154.0418	-7.14
	$C_7H_6N_3O^+$	148.0507	148.0505	-1.35
	$C_6H_5N_3O$	135.0432	135.0427	-3.70
	$C_6H_6N_3^+$	120.0554	120.0556	1.67
	$C_5H_5N_2^+$	93.0451	93.0447	-4.30
	$C_5H_4N^+$	78.0342	78.0338	-5.13
DP-1	**$C_{19}H_{23}ClN_5O3^+$**	**404.1478**	**404.1484**	**1.48**
	$C_{19}H_{22}ClN_5O2$	387.1446	387.1457	2.84
	$C_{19}H_{20}ClN_5O$	369.1336	369.1351	4.06
	$C_{13}H_{16}ClN_2O^+$	251.0955	251.0946	-3.58
	$C_{13}H_{14}ClN2^+$	233.0831	233.084	3.86
	$C_{11}H_{15}N_4O^+$	219.1234	219.124	2.74
	$C_{10}H_{11}ClN_2O$	210.0549	210.0554	2.38
	$C_{10}H_9ClN_2$	192.0459	192.0449	-5.21
	$C_9H_{10}N_3O^+$	176.0818	176.0818	0.00
	$C_8H_9ClN^+$	154.0424	154.0418	-3.90
	$C_7H_6N_3O^+$	148.0509	148.0505	-2.70

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	$C_6H_6N3O^+$	136.0511	136.0505	-4.41
	C_5H_6N2	94.0527	94.0525	-2.13
	$C_5H_4N^+$	78.0335	78.0338	3.84
DP-2	**$C_{19}H_{23}ClN_5O_2^+$**	**388.1518**	**388.1535**	**4.38**
	$C_{19}H_{21}ClN_5O^+$	370.1432	370.1429	-0.81
	$C_{18}H_{19}ClN_5O^+$	356.1289	356.1273	-4.49
	$C_{13}H_{16}ClN_2^+$	235.0988	235.0997	3.83
	$C_{11}H_{15}N_4O^+$	219.1248	219.124	-3.65
	$C_{11}H_{14}ClN2^+$	209.0848	209.084	-3.83
	$C_{10}H_{13}N_4O^+$	205.1084	205.1084	0.00
	$C_{10}H_{11}ClN_2$	194.0615	194.0605	-5.15
	$C_9H_{10}N_3O^+$	176.0823	176.0818	-2.84
	$C_8H_8N_3O^+$	162.0660	162.0662	1.23
	$C_7H_6N_3O^+$	148.0510	148.0505	-3.38
	$C_6H_6N_3O^+$	136.0507	136.0505	-1.47
	$C_6H_6N_3^+$	120.0553	120.0556	2.50
	$C_5H_5N_2^+$	93.0441	93.0447	6.45
	$C_5H_4N^+$	78.0344	78.0338	-7.69
DP-3	**$C_{19}H_{23}ClN_5O_2^+$**	**388.1541**	**388.1535**	**-1.55**
	$C_{19}H_{21}ClN_5O^+$	370.1410	370.1429	5.13
	$C_{18}H_{19}ClN_5O^+$	356.1266	356.1273	1.97
	$C_{13}H_{16}ClN_2^+$	235.0985	235.0997	5.10
	$C_{11}H_{15}N_4O^+$	219.1246	219.124	-2.74
	$C_{11}H_{14}ClN2^+$	209.0849	209.084	-4.30
	$C_{10}H_{13}N_4O^+$	205.1093	205.1084	-4.39
	$C_{10}H_{11}ClN_2$	194.0610	194.0605	-2.58
	$C_9H_{10}N_3O^+$	176.0820	176.0818	-1.14
	$C_8H_8N_3O^+$	162.0658	162.0662	2.47
	$C_7H_6N_3O^+$	148.0511	148.0505	-4.05
	$C_6H_6N_3O^+$	136.0510	136.0505	-3.68
	$C_6H_6N_3^+$	120.0559	120.0556	-2.50
	$C_5H_5N_2^+$	93.0449	93.0447	-2.15
	$C_5H_4N^+$	78.0342	78.0338	-5.13
DP-4	**$C_{17}H_{21}ClN_5O^+$**	**346.1447**	**346.1429**	**-5.20**
	$C_{11}H_{15}N_4O^+$	219.1252	219.124	-5.48
	$C_9H_{10}N_3O^+$	176.0821	176.0815	-3.41
	$C_8H_9ClN^+$	154.0428	154.0418	-6.49
	$C_7H_6N_3O^+$	148.0512	148.0505	-4.73
	$C_6H_5N_3O$	135.0432	135.0427	-3.70
	$C_6H_6N_3^+$	120.0560	120.0556	-3.33
	$C_8H_8N^+$	118.0659	118.0651	-6.78
	$C_5H_5N_2^+$	93.0449	93.0447	-2.15
	$C_5H_4N^+$	78.0343	78.0338	-6.41
DP-5	**$C_{19}H_{24}N_5O_2^+$**	**354.1923**	**354.1925**	**0.56**
	$C_{13}H_{19}N_2O^+$	219.1484	219.1492	3.65

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	$C_{11}H_{15}N_4O^+$	219.1235	219.1240	2.28
	$C_{11}H_{15}N_2O^+$	191.1171	191.1179	4.19
	$C_9H_{10}N_3O^+$	176.0818	176.0818	0.00
	$C_{10}H_{12}NO^+$	162.0906	162.0913	4.32
	$C_7H_6N_3O^+$	148.0499	148.0505	4.05
	$C_6H_5N_3O$	135.0421	135.0427	4.44
	$C_6H_6N_3^+$	120.0550	120.0556	5.00
	$C_5H_5N_2^+$	93.0441	93.0447	6.45
	$C_5H_4N^+$	78.0336	78.0338	2.56
DP-6	**$C_{19}H_{22}Cl_2N_5O^+$**	**406.1205**	**406.1196**	**-2.22**
	$C_{13}H_{17}Cl_2N_2^+$	271.0756	271.0763	2.58
	$C_{11}H_{13}Cl_2N_2^+$	243.0458	243.045	-3.29
	$C_{10}H_{10}Cl_2N_2$	228.0210	228.0216	2.63
	$C_{11}H_{15}N_4O^+$	219.1244	219.124	-1.83
	$C_8H_8Cl_2N^+$	188.0027	188.0028	0.53
	$C_9H_{10}N_3O^+$	176.0814	176.0818	2.27
	$C_7H_6N_3O^+$	148.0514	148.0505	-6.08
	$C_6H_5N_3O$	135.0421	135.0427	4.44
	$C_6H_6N_3^+$	120.0558	120.0556	-1.67
	$C_5H_5N_2^+$	93.0445	93.0447	2.15
	$C_5H_4N^+$	78.0343	78.0338	-6.41
DP-7	**$C_{38}H_{45}Cl_2N_{10}O_2^+$**	**743.3081**	**743.3099**	**2.42**
	$C_{19}H_{23}ClN_5O^+$	372.1566	372.1586	5.37
	$C_{13}H_{18}ClN_2^+$	237.1165	237.1153	-5.06
	$C_{11}H_{15}N_4O^+$	219.1248	219.124	-3.65
	$C_{11}H_{14}ClN_2^+$	209.0834	209.084	2.87
	$C_9H_{10}N_3O^+$	176.0825	176.0818	-3.98
	$C_7H_6N_3O^+$	148.0512	148.0505	-4.73
	$C_6H_5N_3O$	135.0420	135.0427	5.18
	$C_5H_5N_2^+$	93.0444	93.0447	3.22
	$C_5H_4N^+$	78.0341	78.0338	-3.84
DP-8	**$C_{38}H_{45}Cl_2N_{10}O_2^+$**	**743.3110**	**743.3099**	**-1.48**
	$C_{19}H_{23}ClN_5O^+$	372.1577	372.1586	2.42
	$C_{13}H_{18}ClN_2^+$	237.1144	237.1153	3.80
	$C_{11}H_{15}N_4O^+$	219.1228	219.124	5.48
	$C_{11}H_{14}ClN_2^+$	209.0852	209.084	-5.74
	$C_9H_{10}N_3O^+$	176.0811	176.0818	3.98
	$C_7H_6N_3O^+$	148.0498	148.0505	4.73
	$C_6H_5N_3O$	135.0419	135.0427	5.92
	$C_5H_5N_2^+$	93.0441	93.0447	6.45
	$C_5H_4N^+$	78.0333	78.0338	6.41
DP-9	**$C_{38}H_{45}Cl_2N_{10}O_2^+$**	**743.3087**	**743.3099**	**1.61**
	$C_{19}H_{23}ClN_5O^+$	372.1583	372.1586	0.81
	$C_{13}H_{18}ClN_2^+$	237.1156	237.1153	-1.27
	$C_{11}H_{15}N_4O^+$	219.1227	219.124	5.93
	$C_{11}H_{14}ClN_2^+$	209.0832	209.084	3.83

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	$C_9H_{10}N_3O^+$	176.0812	176.0818	3.41
	$C_7H_6N_3O^+$	148.0497	148.0505	5.40
	$C_5H_5N_2^+$	93.0439	93.0447	8.60
	$C_5H_4N^+$	78.0335	78.0338	3.84
DP-10	**$C_{38}H_{45}Cl_2N_{10}O_2^+$**	**743.3097**	**743.3099**	**0.27**
	$C_{19}H_{23}ClN_5O^+$	372.1578	372.1586	2.15
	$C_{13}H_{18}ClN_2^+$	237.1154	237.1153	-0.42
	$C_{11}H_{15}N_4O^+$	219.1220	219.124	9.13
	$C_9H_{10}N_3O^+$	176.0819	176.0818	-0.57
	$C_7H_6N_3O^+$	148.0499	148.0505	4.05
	$C_5H_5N_2^+$	93.0439	93.0447	8.60
	$C_5H_4N^+$	78.0341	78.0338	-3.84

Further major degradation product (DP-10) was isolated and structure was confirmed using NMR experiments. The proton and carbon NMR spectra were given in supplementary information (Fig. Sb to Fig. Se). The proposed structures of protonated DPs are shown in Fig. 2.

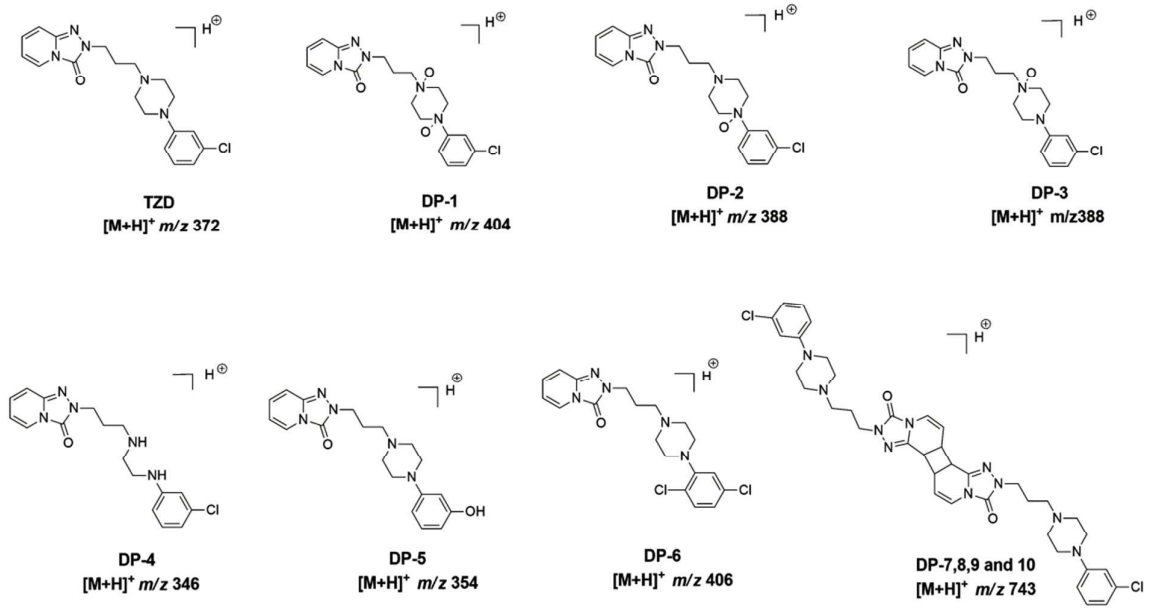

Fig. 2. Chemical structures of protonated TZD and its DPs.

MS/MS of TZD (Rt. 7.2 min)

To elucidate the fragmentation behavior of TZD, the ESI-MS/MS spectrum (Fig. 3a) of its $[M+H]^+$ (*m/z* 372.1583) was examined. The spectrum shows product ions at *m/z* 237, *m/z* 219, *m/z* 209, *m/z* 194, 176, *m/z* 154, *m/z* 148, *m/z* 135, *m/z* 120, *m/z* 93 and *m/z* 78 (Scheme

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1). Product ions at *m/z* 237 and *m/z* 135 can confirm the basic skeleton of 1-(3-chlorophenyl)-4-propylpiperazine and [1,2,4]triazolo[4,3-a]pyridin-3(2H)-one of the drug. The elemental compositions of all the proposed fragment ions have been confirmed by accurate mass measurements and given in Table 1.

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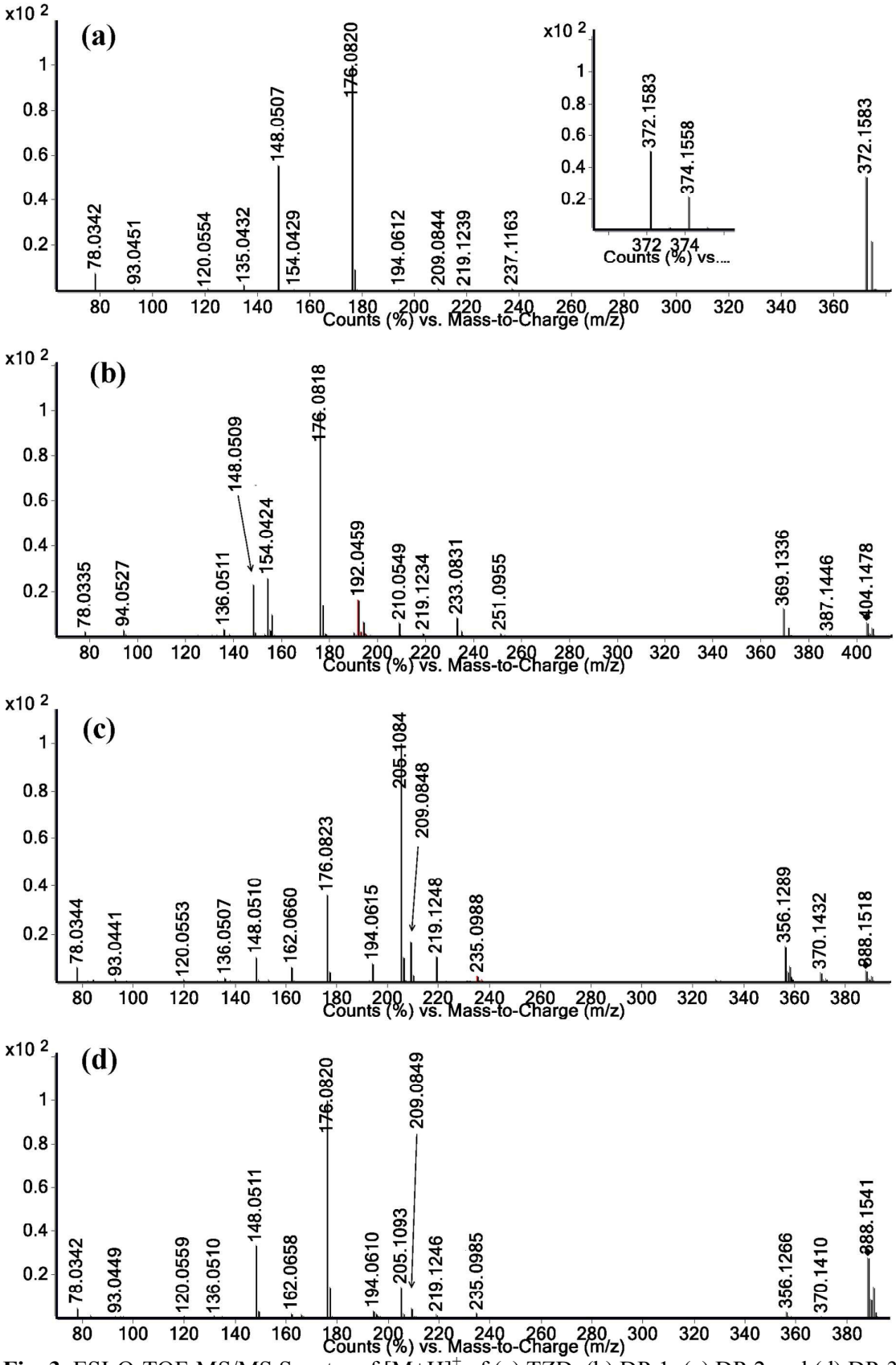

Fig. 3. ESI-Q-TOF-MS/MS Spectra of $[M+H]^+$ of (a) TZD, (b) DP-1, (c) DP-2 and (d) DP-3.

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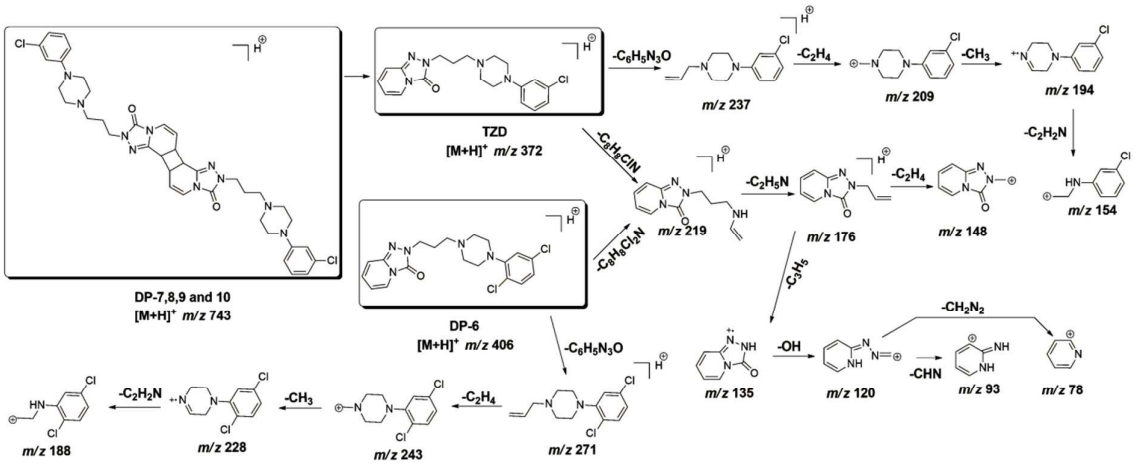

Scheme 1. Proposed fragmentation pathway of $[M+H]^+$ of TZD and DP-6 to DP-10.

MS/MS of degradation products

DP-1 (Rt. 1.6 min)

DP-1 was obtained in oxidative stress degradation. The ESI-MS/MS spectrum of $[M+H]^+$ of DP-1 shows precursor ion peak at *m/z* 404.1502 Da (Fig. 3b) which has 32 Da more molecular mass than the drug suggests the presence of two oxygen atoms in DP-1 structure. Further it was fragmented to give product ions at *m/z* 387, *m/z* 369, *m/z* 251, *m/z* 233, *m/z* 219, *m/z* 210, *m/z* 192, *m/z* 176, *m/z* 154, *m/z* 148, *m/z* 136, *m/z* 94 and *m/z* 78 (Scheme 2). Common fragment ions at *m/z* 219, *m/z* 179, *m/z* 148, *m/z* 135 and *m/z* 78 compared to drug and consecutive loss of OH (*m/z* 404 to *m/z* 387) and H_2O (*m/z* 387 to *m/z* 369) indicates DP-1 is N-oxide degradation product and formed by N-oxidation on the both nitrogen of the piperazine ring of TZD. Based on the above data DP-1 is identified as 1-(3-chlorophenyl)-4-(3-(3-oxo-[1,2,4]triazolo[4,3-a]pyridin-2(3H)-yl)propyl)piperazine 1,4-dioxide, with chemical formula of $C_{19}H_{23}ClN_5O_3$ and mass error of 1.48 ppm.

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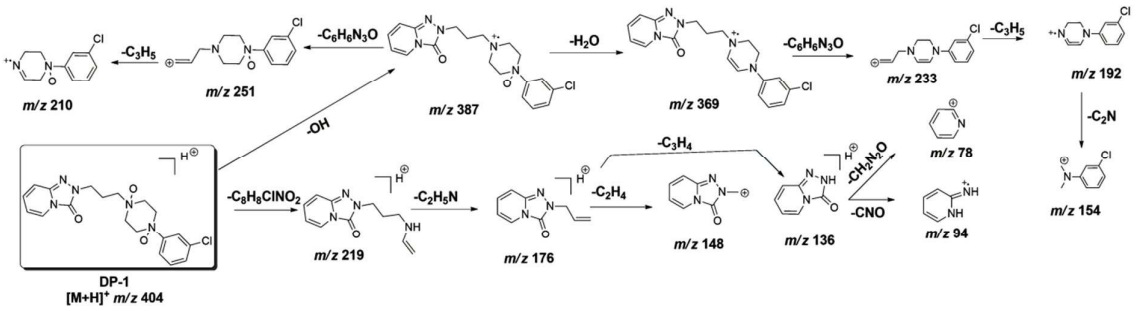

Scheme 2. Proposed fragmentation pathway of $[M+H]^+$ DP-1.

DP-2 (Rt. 3.1 min) and DP-3 (Rt. 4.5 min)

The DP-2 and DP-3 were formed under oxidative stress degradation show 16 Da more molecular mass when compared to drug suggests the presence of oxygen atom in their structures. The ESI-MS/MS spectrum of $[M+H]^+$ of DP-2 (*m/z* 388.1518, (Fig. 3c)) and DP-3 (*m/z* 388.1451, (Fig. 3d)) shows same fragmentation pattern of precursor ions and similar accurate masses of precursor and product ions. Further these precursor ions were fragmented and gave product ions at *m/z* 370 (loss of water), *m/z* 356 (Meisenheimer rearrangement, loss of CH_4O), *m/z* 235, *m/z* 219, *m/z* 209, *m/z* 205, *m/z* 194, *m/z* 176, *m/z* 162, *m/z* 148, *m/z* 136, *m/z* 120, *m/z* 93 and *m/z* 78 (Scheme 3). Based on the obtained product ions and Meisenheimer rearrangement clearly indicates both DPs were N-oxide degradation products which formed by N-oxidation on the each nitrogen of the piperazine ring. The sample was analyzed in full scan APCI-MS mode to determine the site of N-oxidation. The APCI-MS spectrum of DP-2 and DP-3 shows common product ions at *m/z* 372 because of thermal deoxygenation in the APCI source (Fig. 4). In addition, APCI-MS spectrum of DP-3 (Fig. 4b) shows product ions at *m/z* 212 and *m/z* 195 because of Meisenheimer rearrangement which proves DP-3 formed by N-oxidation on the nitrogen attached to the propyl chain while absence of these characteristic product ions in APCI-MS spectrum of DP-2 (Fig. 4a) proves that it was formed by N-oxidation on nitrogen attached to the chlorophenyl ring of drug. Based on the mass fragmentation data DP-2 and DP-3 are identified as 1-(3-chlorophenyl)-4

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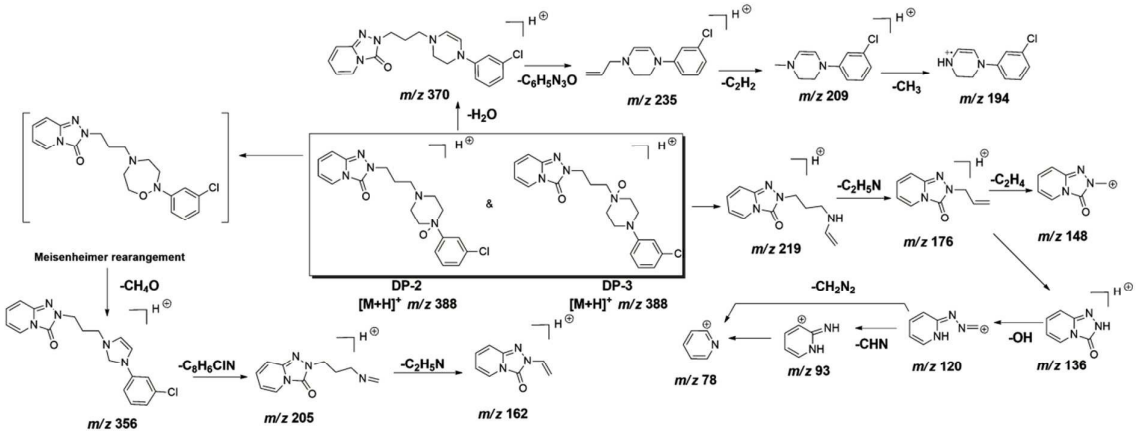

Scheme 3. Proposed fragmentation pathway of $[M-H]^+$ of DP-2 and DP-3.

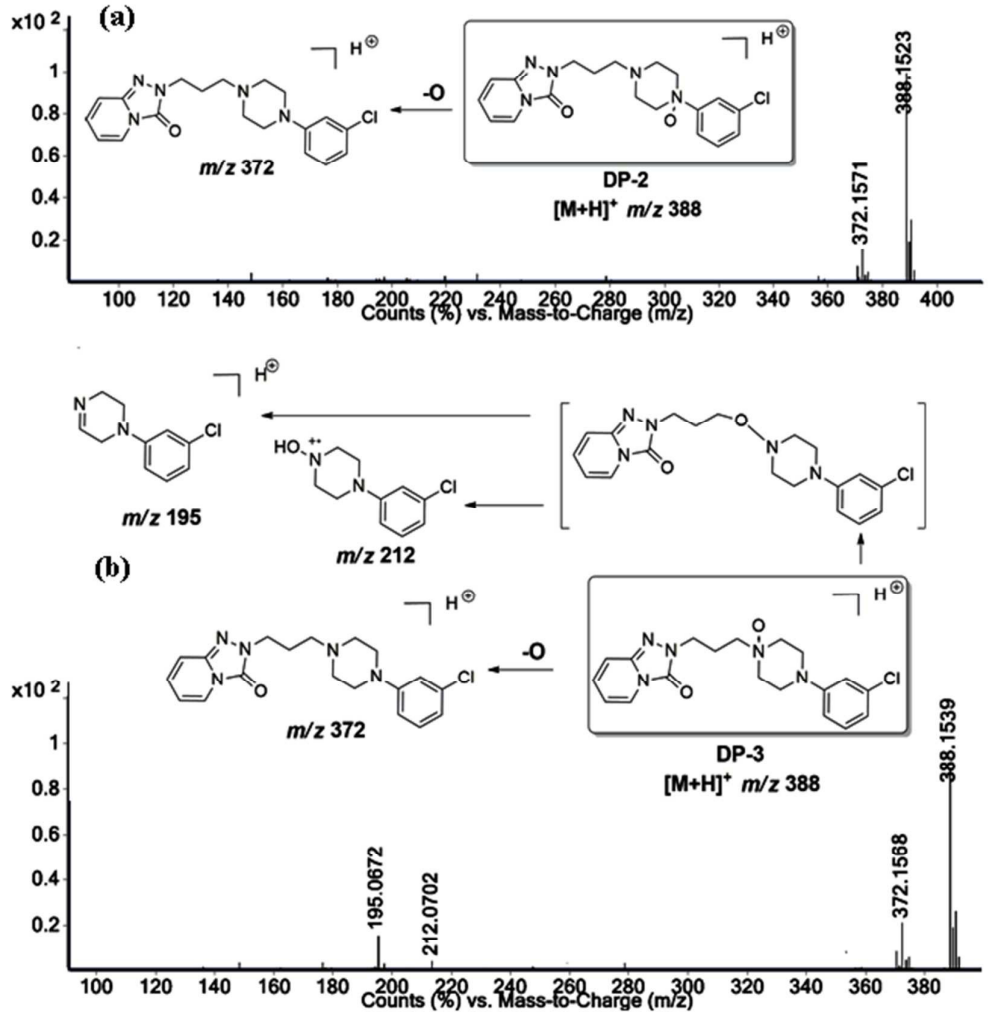

Fig. 4. APCI-MS spectra of $[M+H]^+$ of (a) DP-2 and DP-3.

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-(3-(3-oxo-[1,2,4]triazolo[4,3-a]pyridin-2(3H)-yl)propyl)piperazine 1-oxide and 4-(3-chlorophenyl)-1-(3-(3-oxo-[1,2,4]triazolo[4,3-a]pyridin-2(3H)-yl)propyl)piperazine 1-oxide, respectively. The DP-2 and DP-3 shows same elemental compositions of $C_{19}H_{23}ClN_5O_2$ and mass error of 4.38 and -1.55 ppm, respectively.

DP-4 (Rt. 5.7 min)

The ESI-MS/MS spectrum of protonated DP-4 shows precursor ion peak at *m/z* 346.1447 Da (Fig. 5a). The mass difference of 26.0136 Da compare to drug indicates DP-4 formed by loss of ethylene group from the drug. Further it was fragmented to give product ions at *m/z* 219, *m/z* 176, *m/z* 154, *m/z* 148, *m/z* 135, *m/z* 120, *m/z* 118, *m/z* 93 and *m/z* 78 (Scheme 4). Based on common product ions at *m/z* 219, *m/z* 176, *m/z* 154, *m/z* 148, *m/z* 135, *m/z* 120, *m/z* 93 and *m/z* 78, while absence of product ions at *m/z* 237 and *m/z* 209 compared to drug proves DP-4 formed by loss of ethylene group from piperazine ring of drug molecule. Based on HR-MS data DP-4 was identified as 2-(3-((2-((3-chlorophenyl)amino)ethyl)amino)propyl)-[1,2,4]triazolo[4,3-a]pyridin-3(2H)-one, contain chemical formula of $C_{17}H_{20}ClN_5O$ with calculated mass error of -5.20 ppm.

DP-5 (Rt. 6.9 min)

The ESI-MS/MS spectrum of protonated photolytic degradation product shows precursor ion peak at 354.1923 (Fig. 5b). Further DP-5 fragmented to obtain product ions at *m/z* 219 (*m/z* 219.1484 and *m/z* 219.1235), *m/z* 191, *m/z* 176, *m/z* 162, *m/z* 148, *m/z* 135, *m/z* 120, *m/z* 93 and *m/z* 78 (Scheme 4). The precursor ion peak at *m/z* 354 clearly shows absence of M+2 isotopic peak of chlorine atom (zoomed portion of Fig. 5b) and 18 Da less molecular mass than the drug suggest the presence of -OH in DP-5 structure. MS/MS spectrum of DP-5 shows common fragment ions as that of TZD at *m/z* 219.1235, *m/z* 176, *m/z* 148, *m/z* 135, *m/z* 120, *m/z* 93 and *m/z* 78. The fragment ion at *m/z* 219.1235 indicates the intact portion of [1,2,4] triazolo[4,3-a]pyridine and ruled out the presence of –OH at these portion. The

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product ions at *m/z* 219.1484 and *m/z* 191 are characteristic ions that proved DP-5 formed by replacement of chlorine on phenyl ring with hydroxyl group in the presence of UV-light. Based on the observed data DP-5 was identified as 2-(3-(4-(3-hydroxyphenyl)piperazin-1-yl)propyl)-[1,2,4]triazolo[4,3-a]pyridin-3(2H)-one, having elemental composition of $C_{19}H_{23}N_5O_2$ with calculated mass error of 0.56 ppm.

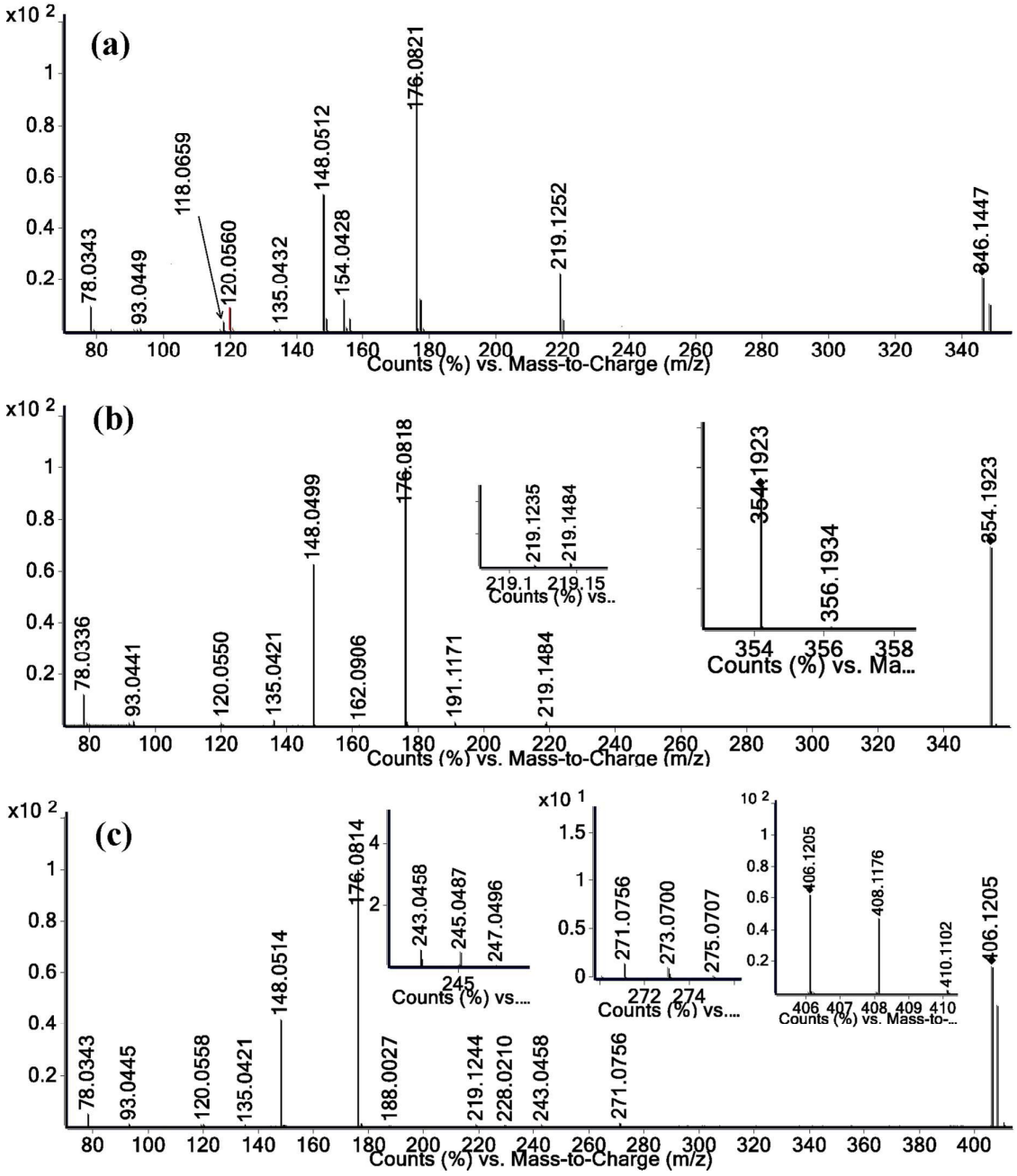

Fig. 5. ESI-Q-TOF-MS/MS Spectra of $[M+H]^+$ of (a) DP-4, (b) DP-5 and (c) DP-6.

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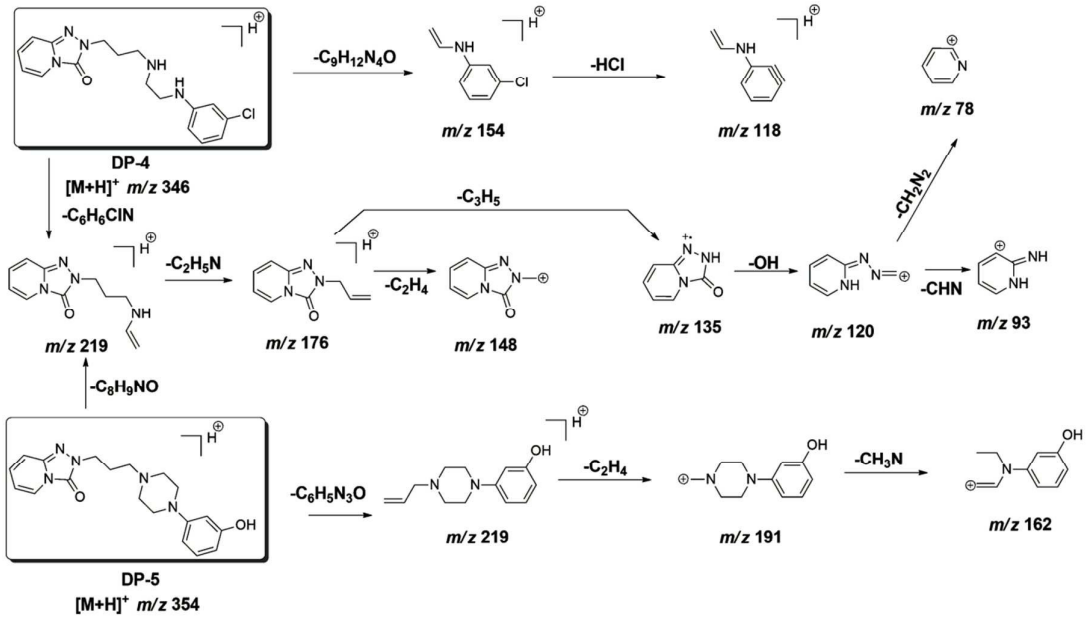

Scheme 4. Proposed fragmentation pathway of $[M-H]^+$ of DP-4 and DP-5.

DP-6 (Rt. 8.3 min)

The ESI-MS/MS of protonated DP-6 shows precursor ion at *m/z* 406.1205 Da (Fig. 5c). Further it was fragmented to obtain product ions at *m/z* 271, *m/z* 243, *m/z* 228, *m/z* 219, *m/z* 188, *m/z* 176, *m/z* 148, *m/z* 135, *m/z* 120, *m/z* 93 and *m/z* 78 (Scheme 1). DP-6 shows common fragment ions at *m/z* 219, *m/z* 176, *m/z* 148, *m/z* 135, *m/z* 120, *m/z* 93 and *m/z* 78 to the drug molecule. The increase in mass of 34 Da when compared to drug indicates the presence of another chlorine atom in chemical structure of DP-6. While product ions at *m/z* 271, *m/z* 243, *m/z* 228 and *m/z* 188 shows 34 units increased in mass when compared to drug product ions at *m/z* 237, *m/z* 209, *m/z* 194 and *m/z* 154, respectively proves chlorination of chlorophenyl ring of drug. Further double chlorine pattern was observed in mass spectrum of drug precursor ion peak and product ions peak at *m/z* 271 and *m/z* 243 proves chlorination of drug (zoomed portion of Fig. 5c). Based on these characteristic features the DP-6 was identified as 2-(3-(4-(3,5-dichlorophenyl)piperazin-1-yl)propyl)-[1,2,4]triazolo[4,3-a]pyridin-3(2H)-one, having elemental composition of $C_{19}H_{21}Cl_2N_5O$ with calculated mass error of -2.22 ppm.

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DP-7 (Rt. 10.2 min), 8 (Rt. 11.0 min), 9 (Rt. 11.4 min) and 10 (Rt. 11.8 min)

The ESI-MS/MS spectrums of $[M+H]^+$ of DP-7, DP-8, DP-9 and DP-10 shows precursor ions peak at *m/z* 743.3081, *m/z* 743.3110, *m/z* 743.3087 and *m/z* 743.3097, respectively (Fig. 6a, 6b, 6c and 6d). Further these DPs were fragmented and the same product ions were obtained at *m/z* 372, *m/z* 237, *m/z* 219, *m/z* 209, 176, *m/z* 148, *m/z* 135, *m/z* 93, and *m/z* 78 (Scheme 1). Similar fragmentation pattern with similar accurate masses for precursor and product ions proves that these are the isomeric degradation products having double molecular mass of TZD (dimers). All these DPs show same elemental composition of a $C_{38}H_{45}Cl_2N_{10}O_2$ and calculated mass error at 2.42, -1.48, 1.61 and 0.27 ppm for DP-7, DP-8, DP-9 and DP-10, respectively. DP-7, DP-8 and DP-9 were observed in small quantity and hence these dimers could not be isolated for structure elucidation. The major dimer (DP-10) was isolated by preparative HPLC and the DP-10 structure was proposed by proton and carbon NMR. ^{1}H and ^{13}C NMR spectrums of DP-10 shows presence of two piperazine ring (15, 16, 18, 19, 15', 16', 18',and 19'), two propyl chain (11, 12, 13, 11', 12' and 13') and two chlorophenyl ring (21, 22, 23, 24, 25, 21', 22', 23', 24' and 25') compare to TZD (Table 2). Also it shows that the two drug molecules were joined at the pyridine rings to form cyclobutyl ring bridge (2,2',3,3' in Table 2) which proves DP-10 structure. Further, the cyclobutyl ring bridge shows δ values of ^{1}H NMR at 2.51 ppm (2H, atoms of 2 and 2') and 2.44 ppm (2H, atoms of 3 and 3') while δ values of 13C NMR shows peaks at 33.24 ppm and 36.18 ppm (for atoms 2 and 2'); 41.62 ppm and 46.57 ppm (for atoms of 3 and 3') proves two drug molecules were joined from the position of 2 and 3 of the pyridine ring of TZD but not from positions of 4 and 5 because presence of nitrogen at position 6 can do deshielding and increase the δ values.

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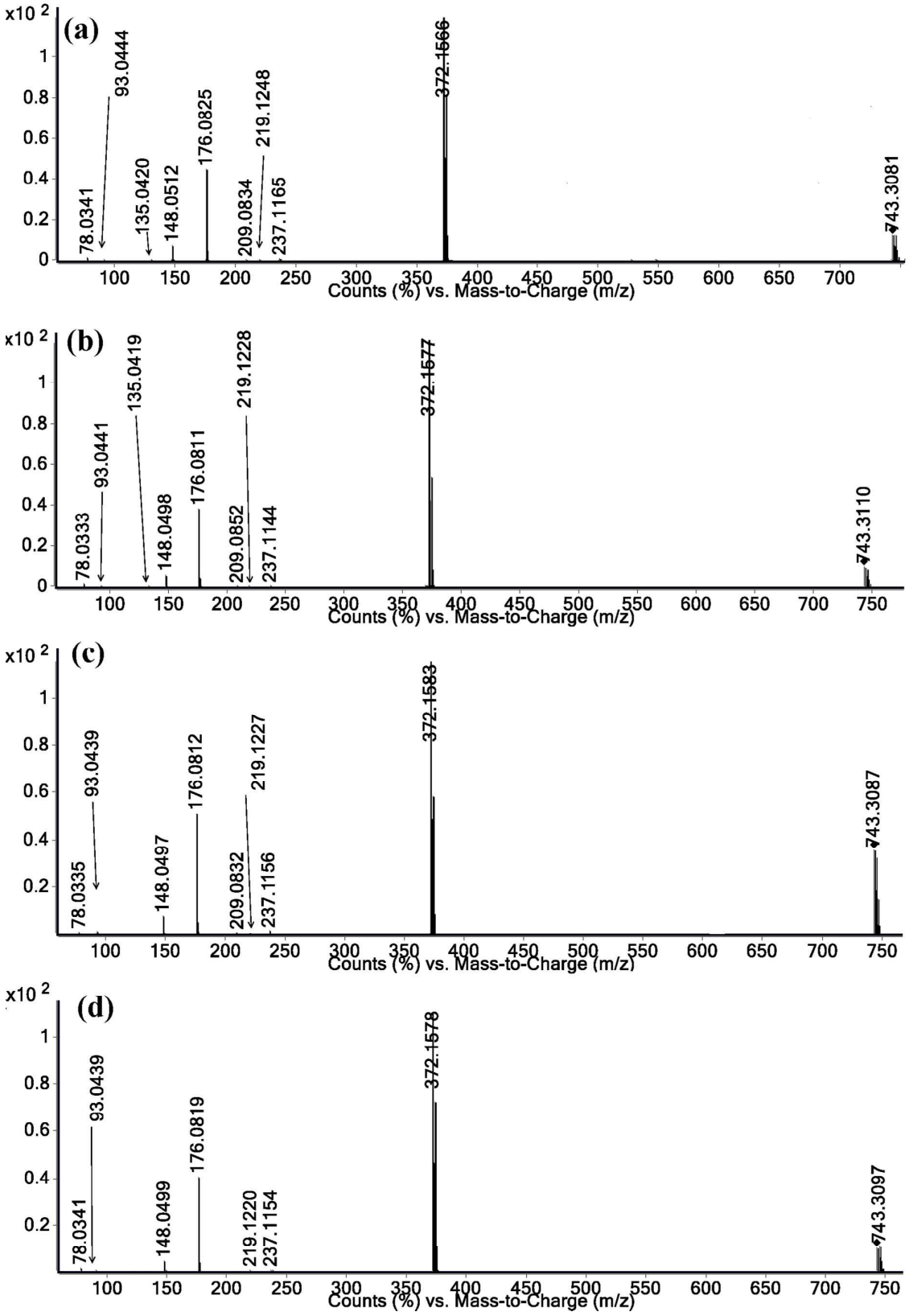

Fig. 6. ESI-Q-TOF-MS/MS Spectra of [M+H]+ of (a) DP-7, (b) DP-8, (c) DP-9 and DP-10.

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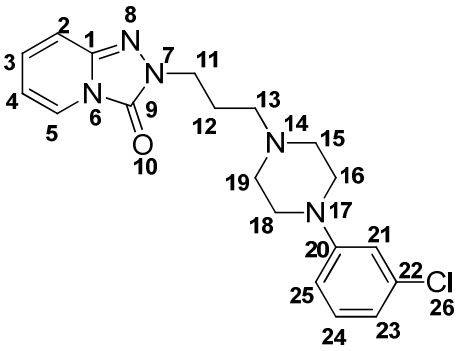
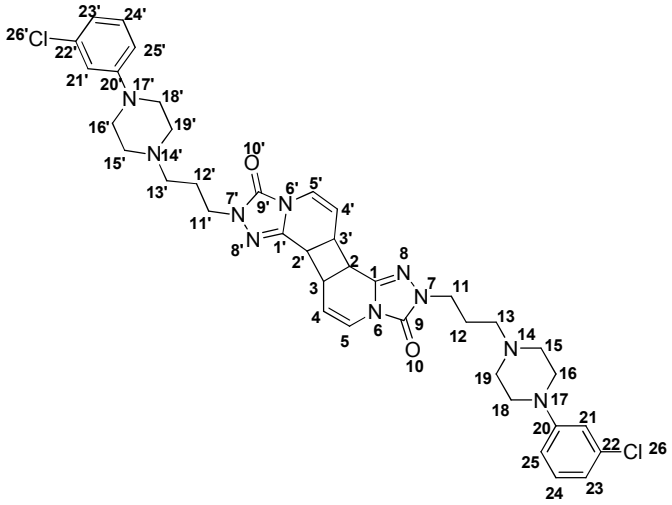
Table 2. NMR chemical shift assignments for TZD and DP-10

TZD



DP-10



Atom position	^{1}H ppm	^{13}C ppm	Atom position	^{1}H ppm	^{13}C ppm
1	-	141.92 (s)	1, 1′	-	139.68 (s) and 137.05 (s)
2	7.09 (1H, d)	123.69 (s)	2, 2′	2.51 (2H, dd)	33.25 (s) and 36.18 (s)
3	7.20 (1H, t)	110.96 (s)	3 , 3′	2.44 (2H, t)	41.62 (s) and 46.57 (s)
4	7.13 (1H, dd)	130.46 (d)	4, 4′	5.20 (2H, m)	106.32 (s)
5	7.75 (1H, d)	115.37 (s)	5, 5′	6.11 (2H, m)	114.05 (d)
6	-	-	6, 6′	-	-
9	-	148.67 (s)	9, 9′	-	149.89 (s),
11	4.17 (2H, t)	55.09 (s)	11, 11′	3.6-4.05 (4H, m)	55.13 (d)
12	2.10 (2H, s)	23.34 (s)	12, 12′	1.89 (4H, m)	25.41 (d)
13	3.73 (2H, m)	43.24 (s)	13, 13′	3.6-4.05 (4H, m)	43.44 (d)
15, 16, 18, 19	3.64 (4H, t), 3.20 (2H, m) and 3.02 (2H, d)	46.39 (s), 50.64 (s) and 51.68 (s)	15, 16, 18, 19, 15′, 16′, 18′ and 19′	3.20 (dd, 8H) and 2.74 (t, 8H)	48.11 (s) and 52.63 (d)
20	-	150.23 (s)	20, 20′	-	151.94 (s) and 151.41 (s)
21	6.93 (1H, d)	117.32 (s)	21, 21′	6.85 (2H,m)	115.56 (s) and 120.32 (s)
22	-	135.20 (s)	22, 22′	-	134.97 (s) and 130.33 (s)
23	6.81 (1H,d)	121.64 (s)	23, 23′	6.78 (2H, m)	119.64 (d)
24	6.53 (1H, t)	115.11 (s)	24, 24′	6.78 (2H, m)	115.97 (d)
25	6.93 (1H, d)	130.46 (d)	25, 25′	7.14 (2H, m)	130.11 (d)

Degradation pathway of the drug

The chemical structure of drug contains triazolo-pyridine ring, propyl chain, piperazine ring and chlorophenyl ring. Literature shows that the piperazine ring is more prone to be oxidized to obtain N-oxide degradation products [10]. In oxidative stress degradation condition, by attack of H_2O_2 on tertiary nitrogens of piperazine ring DP-1 to DP-3 were formed as N-oxides. DP-1

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was formed by N-oxidation on the both nitrogen of the piperazine ring of TZD. The DP-2 was formed by N-oxidation on the nitrogen attached to the chlorophenyl ring of drug and DP-3 was formed by N-oxidation on the nitrogen attached to the propyl chain of drug. The DP-4 was formed in acidic hydrolysis by loss of ethylene group from the piperazine ring of TZD [10]. DP-5 was formed by loss of chlorine atom from chlorophenyl ring followed by hydroxylation in the aqueous environment under UV light. DP-6 was formed by electrophilic substitution of chlorine on chlorophenyl ring of TZD under acidic stress condition [25]. The DP-7, DP-8, DP-9 and DP-10 were isomeric dimer degradation products of drug. They were formed by joining of two drug molecules at the pyridine rings to form cyclobutyl ring bridge.

Method validation

Stability indicating UHPLC assay method was validated for specificity, limit of detection, limit of quantification, linearity, accuracy, precision and robustness according to ICH-guideline Q2 (R1) and USP general chapter for method validation. [23-24]

Specificity

The specificity of the developed method was demonstrated by checking peak purity of chromatographic peaks using photodiode array detector (PDA) and additionally proved by the mass detector. All chromatographic peaks (TZD and DPs) were well resolved from each other and purity angle values were found to be within purity threshold values which unambiguously prove the specificity of the developed method.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Different concentration of drug standard solutions at 0.05, 0.1, 1.0, 1.5 and 2 µg mL^{-1} were injected to find the LOD and LOQ concentration values of the drug. Based on observed signal-to-noise (S/N) ratios, LOD and LOQ concentrations were found to be 0.035 µg mL^{-1} and 0.11 µg mL^{-1}, respectively. Replicated injections (n=5) were analyzed to confirm obtained LOD and LOD concentrations of the drug.

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Linearity

The linearity of UHPLC method was determined on five different concentrations (80-120 µg mL^{-1}) of TZD working standard solutions in triplicate (n=3). Plotted standard calibration curve shows splendid relationship between concentrations of drug on x-axis and respective mean peak areas on y-axis. The obtained result shows acceptable values of determination coefficient (r^2) of 0.999 and linear regression equation of 13551 x - 18300. Hence, the developed method shows linear response over performed concentration range.

Accuracy

Accuracy of the method was determined by spiking three known concentrations of standard drug solutions into stressed solution in triplicate (n=3). The recovery of spiked drug was measured from difference peak areas of fortified and unfortified stressed samples. The recovery and % RSD of TZD was was found to be 99.83 to 100.15 % (Table 3) and below 1 percentage, respectively proves the accuracy of proposed UHPLC method.

Precision

Intra- and inter-day precisions were determined using three different concentrations of 80, 100 and 120 µg mL^{-1}, on the same day (*n= 3*) and consecutive days (*n= 3*). The observed values of % RSD for intra and inter-day precisions were found to be below 1% which proves developed UHPLC method was sufficiently precise (Table 3).

Table 3. Accuracy and Precision data for TZD

Concentration TZD added (µg mL^{-1})	**Concentration TZD obtained**	**% Recovery**	**Intra-day precision (n=3)**	**Inter-day precision (n=3)**
	(µg mL^{-1}) ± SD; %RSD		**Mean TZD concentration (µg mL^{-1}) ± SD; %RSD**	
80	79.86 ± 0.37; 0.47	99.83	79.75 ± 0.42; 0.53	79.86 ± 0.45; 0.56
100	100.04 ± 0.31; 0.31	100.04	100.03 ± 0.32; 0.32	100.10 ± 0.35; 0.35
120	120.18 ± 0.15; 0.12	100.15	120.26 ± 0.20; 0.17	120.4 ± 0.26; 0.21

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Robustness

Robustness study was carried out on the drug degradation samples by making deliberate changes to method variables like flow rate (0.05 mL min^{-1}), column oven temperature (±5 °C), mobile phase pH (±0.3 unit) and organic percentage (±2 %). Obtained peak areas were used to calculate % RSD and it was found to be below 1 %. Other assay parameters like resolution between critical pair of DP-8, DP-9 and DP-10, tailing factor and USP plate count of drug peak were found to be within the limits which proved the robustness of the developed method.

Conclusion

The degradation behavior of Trazodone under various stress degradation conditions was studied. The drug was found to be stable under basic hydrolysis, neutral hydrolysis and dry heat while it was degraded under acid hydrolysis, oxidative stress and photolytic degradations. A total of ten DPs were obtained and were well separated using developed UHPLC method. The DPs were characterized using UHPLC-ESI-QTOF-MS/MS experiments. The site of N-oxidation in N-oxide degradation products (DP-2 and DP-3) was confirmed by using APCI-MS. The major degradation product (DP-10, dimer of drug) was isolated by preparative HPLC and further structure was confirmed by using proton and carbon NMR. Formation of interesting DPs such, N-oxides in oxidative stress degradation (DP-1 to DP-3), loss of an ethylene group from the piperazine ring in acidic hydrolysis (DP-4), substitution of chlorine of the chloro-phenyl ring by hydroxyl group in the presence of UV-light (DP-5), chlorination of the chlorophenyl ring in acidic hydrolysis (DP-6) and formation of dimers of TZD in the presence of UV-light (DP-7 to DP-10) were observed. The proposed method was validated as per ICH guidelines. The given method can be used in routine analysis and stability study of drug in quality control laboratories.

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Conflicts of interest

Authors have declared no conflicts of interest.

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Graphical Abstract