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Development of stability indicating UPLC method for terconazole and characterization of acidic and oxidative degradation products by UPLC-Q-TOF/MS/MS and NMR

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Abstract

Terconazole, a triazole antifungal drug is used to treat infections in the form of cream or suppositories. A simple, rapid, precise and accurate ultra high performance liquid chromatography method has been developed for the quantitative determination of the terconazole drug substance in the presence of process impurities and degradation products. The method showed adequate separation of terconazole, process impurities and degradation products on the CSH C_{18} (100 × 2.1 mm, 1.7 µm) column. The mobile phase used was ammonium acetate (10 mM; pH 7.5) and acetonitrile as organic modifier. Terconazole was subjected to forced degradation studies and found to be degraded in acid hydrolytic and oxidative conditions whereas; it was stable in basic, neutral, photolytic and thermal conditions. LC/electrospray ionization or and LC/atmospheric pressure chemical ionization coupled with quadrupole time-of-flight mass spectrometer has have been utilized for identification and structural characterization of the degradation products. The major oxidative degradation products were isolated on preparative HPLC and structures were confirmed using ^{1}H NMR and ^{13}C NMR. The probable mechanistic explanation is given for the formation of degradation products. The method was validated in terms of specificity, linearity, accuracy, precision and robustness as per International Conference on Harmonisation guideline.

Keywords: terconazole; process impurities; forced degradation; degradation products; characterization; UPLC-Q-TOF/MS-MS.

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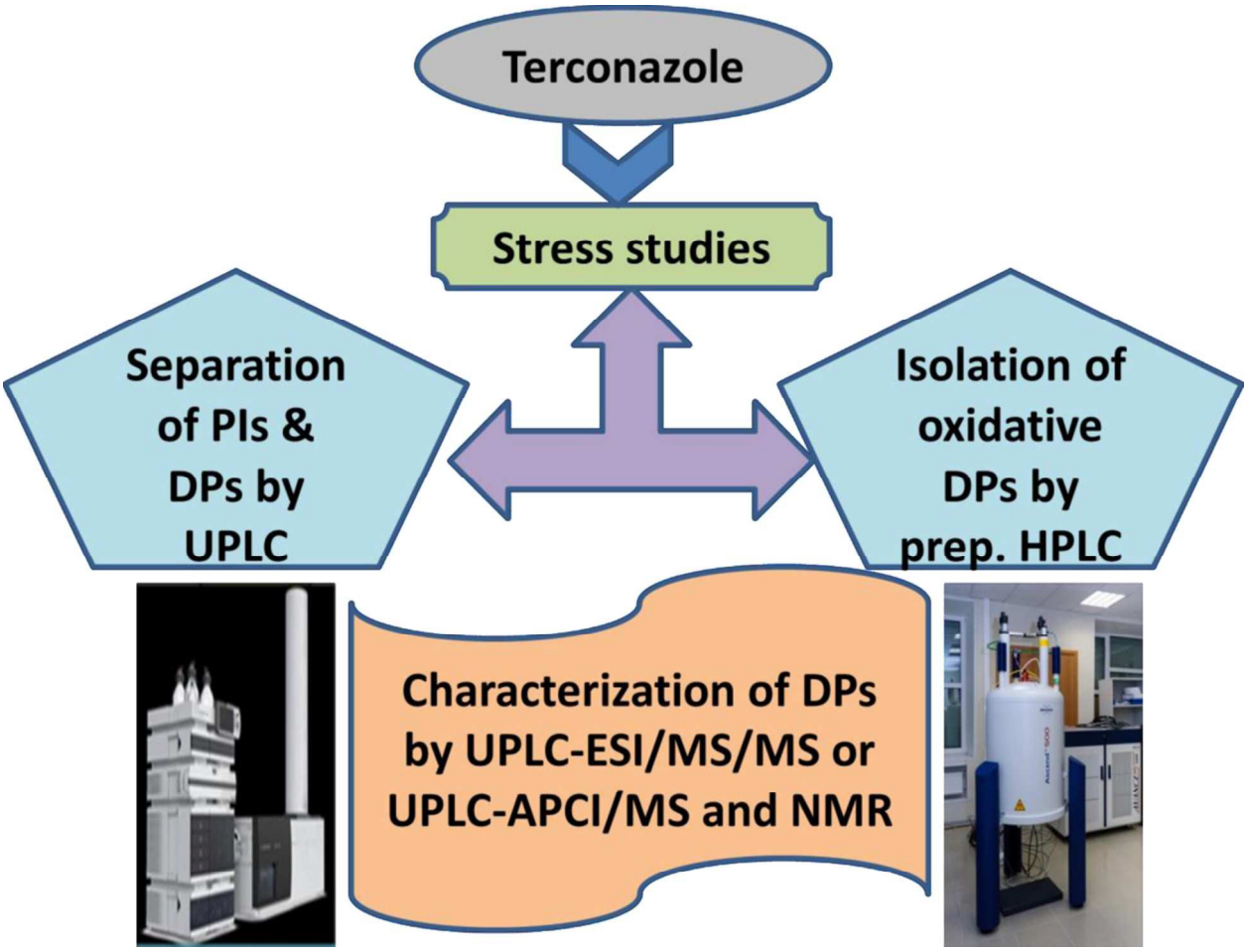
Graphical abstract



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Introduction

Terconazole (TER) 1-[4-[[(2*S*,4*S*)-2-(2,4-Dichlorophenyl)-2-(1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-4-propan-2-yl-piperazine, is an antifungal drug with molecular formula of $C_{26}H_{31}Cl_2N_5O_3$. TER is a triazole antifungal drug, primarily used for the fungal and yeast infections in vagina in the form of vaginal cream or suppository.[1, 2] The drug may undergo degradation which result in a loss of potency and initiate the possible adverse effect due to formation of degradation product or impurities. Stability testing guidelines Q1A (R2) developed by International Conference on Harmonization (ICH) involve identification of degradation products, degradation pathways and intrinsic stability of drugs.[3] Intrinsic stability of drugs is determined by applying the stress under hydrolytic, oxidative, thermal and photolytic conditions as described in ICH guideline, Q1A (R2). To ensure the quality and safety of the drug, the identification and characterization of the degradation products (DPs) is of prime importance which helps us to establish degradation pathways of the drug.

Based on the comprehensive literature search, it was observed that, a few reports are available on TER like; micellar liquid chromatographic,[4] HPTLC determination (from biological matrix) method.[5] Maria Castro *et al.* have investigated the enanatiomeric separation of ketoconazole and terconazole.[6] Nadia Bounoua *et al.* reviewed achiral and chiral separations of antifungal drugs including TER by HPLC and CE.[7] As per the best of our knowledge, no information is exists in the literature about the stability indicating UPLC method for the separation of TER from its PIs and DPs. Also there is no information available on the forced degradation behavior of the drug. This dearth of information prompted us to develop a method which can be useful for the separation of the drug from its PIs/DPs and characterization of DPs using UPLC-ESI/MS/MS and NMR. Nowadays, liquid chromatography electrospray ionization

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tandem mass spectrometry (LC-ESI/MS/MS) and NMR has been widely used for the identification and characterization of forced degradation products (DPs) and impurities.[8-10]

So the endeavour of the present study was to (1) carry out stress degradation of the TER under various stress conditions, (2) develop selective RP-UPLC method for TER, PIs and DPs, (3) isolate the major oxidative DPs using preparative HPLC (4) characterize the degradation products using either UPLC-ESI/MS/MS or UPLC-APCI/MS and NMR (5) validate the developed method according to the ICH prescribed guideline.[11]

Experimental

Drug and reagents

TER and PIs (Imp-1, Imp-2, Imp-3 and Imp-4) were obtained as gratis sample from Nosch labs Pvt. Ltd, Hyderabad, India. Analytical reagent (AR) grade ammonium acetate, ammonium formate (HPLC grade), formic acid (FA) and acetic acid (AA) were purchased from S.D. Fine Chemicals (SDFCL) (Mumbai, India). Analytical reagent (AR) grade sodium hydroxide (NaOH) and laboratory reagent (LR) grade hydrogen peroxide (H_2O_2) was obtained from SDFCL Mumbai, India. Hydrochloric acid (HCl) was purchased from FCL Ahmadabad, India. LC-MS CHROMASOLV grade methanol (MeOH) and acetonitrile (ACN) were obtained from Sigma-Aldrich (Bangalore, India). Water used for the present study was purified by the Milli Q purification system (Millipore, Bedford, MA, USA). Tetramethylsilane (TMS) and $CDCl_3$ were purchased from Sigma-Aldrich (Bangalore, India) and Euriso-top (France), respectively.

Instrumentation

The chromatographic method development was carried out by using Acquity UPLC-H class system from Waters (Milford, MA, USA). The instrument was equipped with bio-quaternary

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gradient pump with in-line degasser, and flow through needle design integral sample manager (SM-FTN). A column compartment with temperature control and a photodiode array (PDA) detector was used. The system was controlled by Empower-3 software. Thermal stability study was performed in a dry air oven (Mack pharmatech Pvt. Ltd., Mumbai, India). Photostability chamber (Mack equipment, Thane, India) was used for photo degradation study with temperature range of 20 °C to 50 °C. The photo stability chamber (MK2-40P) was equipped with eight UV and visible light tubes. All pH measurements were done on pH meter (Metrohm Schweiz AG, 780 pH meter, Germany). Other equipments used includes, a Power Sonic405 ultrasonicator from Hwashin Technology Co., (Seoul, Korea) and Sartorius balance (CD 225 D, 22308105 Germany) was used to dissolve the samples and to weigh the samples, respectively.

UPLC-MS analysis of TER was performed using an Agilent 1200 infinity series UPLC instrument (Agilent Technologies, USA) attached to a quadrupole time-of flight (Q-TOF) mass spectrometer (Q-TOF LC-MS G6540B series, Agilent Technologies, USA) equipped with either an electrospray ionization (ESI) source or an atmospheric pressure chemical ionization (APCI) source. The data acquisition was under the control of Mass-Hunter Workstation software. The typical operating source conditions for MS scan of TER, PIs and DPs in positive ESI mode were optimized as, fragmentor voltage 144 V, capillary Voltage 3500 V, capillary temperature 250 °C, skimmer Voltage 65 V, drying gas (N_2) pressure 40 psi (325 °C, 10 L min^{-1}), Scan range 100-1000, scan rate 5 spectra/sec. In APCI, the vaporizer temperature was 400 °C and the source current was set at 3µA. All other source parameters were same as used in ESI. Collision-induced dissociation (CID) experiments were conducted by keeping MS1 static, the precursor ion of interest was selected using the quadrupole analyzer, and the product ions were analyzed using

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the TOF analyzer. Ultrahigh pure nitrogen gas was used as collision gas. All the spectra were recorded under identical experimental conditions.

NMR experiments (^{1}H and ^{13}C) were carried out using 500 MHz NMR (AVANCE III HD-500, Bruker, Switzerland) spectrometer. TMS and $CDCl_3$ were used as an internal standard and solvent for NMR experiments, respectively. The chemical shift values were reported on the δ scale in ppm.

Stress degradation studies

Stress degradation studies were carried out with TER drug substance (0.2 mg mL^{-1}) to identify the possible DPs and validate the developed stability indicating analytical method. Specificity of the developed method was conducted in the presence of its DPs. TER was subjected to forced degradation studies under hydrolytic conditions like acidic (1N HCl), basic (3N NaOH) and neutral (pure water) for 30 h, 4 days and 5 days, respectively at 80 °C. Oxidative studies were carried out at room temperature in 0.6% hydrogen peroxide for 1 h. Thermal degradation was performed by keeping the sample evenly spread in a petri plate for 7 days in hot air oven at 100 ºC. Photolytic stress studies were carried out by exposing a solid layer of TER evenly spread in glass petri plate to UV (200Wh/m^2) and fluorescent light (1.2×10^6 lux h) in photo stability chamber for 7 days. Suitable controls were kept in photo stability chamber under the dark with the aluminium foil wrapped for the conditions mentioned above. The optimized stress degradation studies for all the conditions are given in **Table S1**.

Sample preparation

TER and its impurities were poorly soluble in the water, hence it was dissolved in the diluent (water: ACN, 50:50 v/v). The stock solution of TER and each impurity were prepared in the

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diluent at the concentration of 1.0 mg mL^{-1} and 0.2 mg mL^{-1}, respectively. The sample solution of the TER containing 500 µg mL^{-1} and 200 µg mL^{-1} were prepared from the stock solution for the related substances and assay determination, respectively. TER (500 µg mL^{-1}) spiked with 0.1% of PIs *i.e;* Imp 1, methyl 2-(2-(bromomethyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)benzoate, Imp 2, (2-((1H-1,2,4-triazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)methyl methanesulfonate, Imp 3, 4-(4-isopropylpiperazin-1-yl)phenol, Imp 4, 1-(4-methoxyphenyl)piperazine (**Fig. S1**) was used throughout study. The stock solutions of TER and PIs were diluted adequately to perform precision, accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ) and robustness. The stress samples were withdrawn at suitable time intervals. Neutralization of the stressed samples of acid and base was done with suitable strengths of NaOH and HCl, respectively. All the stressed samples were diluted with the diluent to the final concentration of 200 µg mL^{-1} and filtered through nylon-66 membrane syringe filters (0.22 µm) before analysis.

The sample solution of TER was prepared by mixing terconazole cream equivalent to 1mg mL^{-1} with the diluent. The solution was filtered through 0.22 µ nylon filter and clear solution was diluted five times with diluent to perform assay of TER.

Method validation

The developed UPLC method was validated for assay and related substances as per ICH guideline.[11] The accuracy of assay for TER (200 µg mL^{-1}) was calculated in triplicate at concentrations 100, 200 and 300 µg mL^{-1} (50-150%) on drug substance and recoveries were calculated for each sample. The recoveries of the PIs were evaluated at three concentration levels *i.e;* 0.05%, 0.1% and 0.15% of the nominal concentration of TER (500 µg mL^{-1}). The precision of the assay was determined by injecting six samples at assay concentration (200 µg mL^{-1}) and

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RSD (%) values of the results was calculated. The repeatability of the related substance (RS) method was determined by injecting six replicates of TER spiked with 0.1% each of the PI. Intra-day precision was evaluated by injecting 500 µg mL^{-1} TER (*n=3*) spiked with 0.1% of PI. The similar experiments were carried out on different days by different analyst to determine inter-day precision. The peak area RSD (%) was calculated for PIs. Linearity for the assay of TER was analyzed at seven different concentrations ranging from 50, 100, 150, 200, 250, 300, 350 µg mL^{-1}. Linearity test samples for the related substances were prepared by adequately diluting the stock solution of the PIs. The linearity for all four PIs were determined at seven concentration levels ranging from 50% to 200% (*i.e;* 0.05, 0.075, 0.1, 0.125, 0.15, 0.175 and 0.2%) with respect to 500 µg mL^{-1} of TER. The peak area *versus* concentration of TER and PIs were treated by linear regression analysis. The correlation coefficients, Y-intercepts and slopes of calibration curve were determined. The LOD and LOQ of TER and PIs were determined at signal-to-noise ratio of 3:1 and 10:1, respectively, by analyzing dilute solutions of known concentration. The robustness study was conducted to determine the effect of small but deliberate change in optimized LC method. The factors evaluated were mobile phase flow rate by ±0.03 mL min^{-1}, column oven temperature by ±5 °C, mobile phase pH by ±0.2 units. The resolution between TER and all four impurities were determined.

Isolation of DPs by preparative HPLC

The chromatographic method was transferred from UPLC to HPLC for isolation of the DPs. The preparative isolation of the major oxidative degradation products were carried out on preparative HPLC from Waters (Milford, MA, USA) equipped with 515 HPLC pumps and 2489 UV–visible detector. The separation of DPs was carried out on Waters X bridge C18 column (250 × 19 mm × 5 µm) with ammonium acetate (pH 7.5; 10 mM) and ACN in gradient elution mode. The

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gradient program was set as 0/10, 2/10, 10/60, 15/70, 17/10, 20/10 (T_{min}/%ACN). The flow rate and injection volume was 10 mL min^{-1} and 1 mL, respectively. The detection wavelength was fixed at 227 nm. The oxidative DPs (DP5, DP6 and DP7) were isolated for the structural confirmation by NMR. The drug (2000 µg mL^{-1}) was allowed to degrade in 6% peroxide (H_2O_2) solution for 4 h to get more percentage of the DPs. The fraction of the DPs were collected by multiple injections into preparative HPLC and pooled together. Pooled fractions were concentrated at 30 °C using rotary evaporator to remove ACN and further lyophilized to get the solid compound. All the DPs were found to be pure with chromatographic purity >97%. The solid form of the DPs were dissolved in $CDCl_3$ and submitted for NMR analysis.

Results and Discussion

Optimization of Chromatographic separation

The objective of the present study was to develop stability indicating UPLC method to separate TER from the PIs as well as from the DPs formed under stress conditions. For the method development studies TER was spiked with PIs (Imp 1 to Imp 4). Simultaneously, forced degradation samples were also analyzed in UPLC. The Acquity CSH C_{18} column (100 x 2.1 mm, 1.7 µm) was found to be suitable for the purpose. The method development was initiated by using 0.1 % FA and ACN, where Imp1 and Imp2 got separated from the TER as well as from each other but the specified method does not produce adequate resolution of Imp 3 and Imp 4. MeOH was used instead of ACN as an organic modifier; but there was no significant improvement in the resolution between Imp3 and Imp4. Further, trials were conducted by using different aqueous buffers like ammonium formate (10 mM), ammonium acetate (10 mM) with organic modifiers like ACN and MeOH. By using ammonium formate (10 mM) with ACN, TER

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and PIs were well separated with optimum resolution but when the same method was applied to stress degradation samples, Imp3 and DP1 (acidic DP) were eluted at the same retention time and oxidative DPs (DP6 and DP7) were not resolved. Ammonium formate and ammonium acetate were tried at different pH values with organic modifiers (ACN and MeOH), so as to get best separation of the analytes. Finally, TER, PIs and all the DPs were well separated with optimum resolution with no interference from the excipients by using mobile phase composed of volatile buffer (A) ammonium acetate (pH 7.5; 10 mM) and (B) ACN as organic modifier employing a gradient program of 0/20, 4/40, 7/80, 10/80, 12/20, 14/20 (T_{min}/%ACN) for 14 min (**Fig. S2a-e**). Column temperature and mobile phase flow rate was maintained at 30 °C and 0.25 mL min^{-1} respectively. Injection volume was 1.0 µL. Prior to sample injection, column was equilibrated with the mobile phase at the initial gradient composition for 10 min. Mobile phase was filtered through 0.22 µm, Millipore Nylon-66 filter. All the samples were analyzed using a PDA detector and chromatograms were extracted at 227 nm to detect PIs and the possible degradation peaks. The same method was transferred for UPLC-MS studies by optimizing all the MS parameters as discussed in experimental section.

Method validation

Specificity of the method was determined by estimating the purity angle and purity threshold of TER and its PIs using a PDA detector. It was noticed that purity angle was lesser than purity threshold for all PIs and TER in presence of the DPs, indicates the specificity of the method. Accuracy of the developed method was determined by the recovery experiments in triplicate at three levels. Recovery of TER and all four PIs was found between 98.09-100.73 and 93.04-100.05, respectively (**Table S2**). The RSD (%) values for the repeatability of TER assay and PIs (Imp 1 to Imp 4) was found to be within 2.0 %. The RSD (%) results of intermediate precision

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(intra-day and inter-day) for TER and PIs are less than 3% (**Table S2**). These results prove that the method was precise. Linearity for TER and PIs was determined by plotting calibration curve over tested range and linear regression analysis of peak area *versus* concentration gives good linearity. Slope, intercept and correlation coefficient data was presented in **Table S2**. The LOD and LOQ values for the TER and PIs (Imp 1 to Imp 4) are given in **Table S2**. Robustness of the method was studied by deliberate change in chromatographic parameters. In all experiments TER and PI peaks were found to be well resolved with resolution more than 2 and elution order of the analytes remain unchanged. RSD (%) value for all the parameters was within the limits indicates the robustness of method.

Degradation behavior of TER

The behavior of TERunder various stress degradation conditions were studied by employing UPLC. The drug was found to be stable under basic, neutral, thermal and photolytic stress conditions, while it was found to be degraded under acid hydrolytic (**Fig. S2d)** and oxidative conditions **(Fig. S2e).** Total of seven DPs were identified and characterized by UPLC-ESI/MS/MS and their elemental compositions are depicted in Table S3. The structures of oxidative DPs were confirmed by NMR analysis.

The drug was refluxed in 0.5 N HCl for 8 h at 80 °C, where it was found to be stable. Whereas four DPs (DP1 to DP4) were observed in 1N HCl refluxed at 80 °C for 30 h **(Fig. S2d).** A total of four DPs (DP2, DP5 to DP7) were formed in presence of 0.6% H_2O_2 after 1 h in the dark room at 25 °C indicates that the drug is sensitive to oxidative condition **(Fig. S2e).** No degradation products were formed in basic hydrolytic, neutral hydrolytic, thermal and photo solid conditions.

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UPLC-Q-TOF/MS/MS study of TER and its DPs

ESI/MS/MS of TER [$(M+H)^+$, *m/z* 532]

The UPLC-ESI/MS/MS line spectrum of TER $[M+H]^+$ ion (*m/z* 532) is shown in **Fig. 1a.** It clearly showed product ions at *m/z* 490 (loss of C_3H_6 from *m/z* 532), *m/z* 447 (loss of $C_5H_{11}N$ from 532), *m/z* 312 (loss of $C_{13}H_{20}N_2O$ from *m/z* 532), *m/z* 277 (loss of $C_{10}H_7Cl_2N_3O$ from 532), *m/z* 259 (loss of H_2O from *m/z* 277), *m/z* 256 (loss of C_3H_4O from 312), *m/z* 235 (loss of C_3H_6 from *m/z* 277), *m/z* 219 (loss of C_3H_6O from *m/z* 277), *m/z* 218 (loss of NH_3 from *m/z* 235), *m/z* 217 (loss of H_2O from *m/z* 235), *m/z* 205 (loss of $C_3H_4O_2$ from *m/z* 277), *m/z* 192 (loss of $C_5H_{11}N$ from *m/z* 277), *m/z* 178 (loss of C_3H_7N from *m/z* 235), *m/z* 177 (loss of C_3H_7 from *m/z* 219), *m/z* 163 (loss of C_3H_6 from *m/z* 205), *m/z* 148 (loss of CH_3N from *m/z* 177), *m/z* 136 (loss of C_2H_3N from *m/z* 177), *m/z* 111 ($C_6H_{11}N_2^+$) and *m/z* 86 ($C_5H_{12}N^+$). The elemental compositions of all these ions have been confirmed by accurate mass measurements **(Table 1)**. The formation of abundant product ion *m/z* 277 can be explained by the loss of 1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethanol from *m/z* 532 which indicates the presence of dichlorophenyl moiety and triazole ring in TER. The formation of *m/z* 447 can be explained by the loss of propylaziridine from the TER (*m/z* 532) which confirms the presence of piperazine moiety in TER.[12] Proposed mass fragmentation pathway of TER is depicted in **Scheme 1**.

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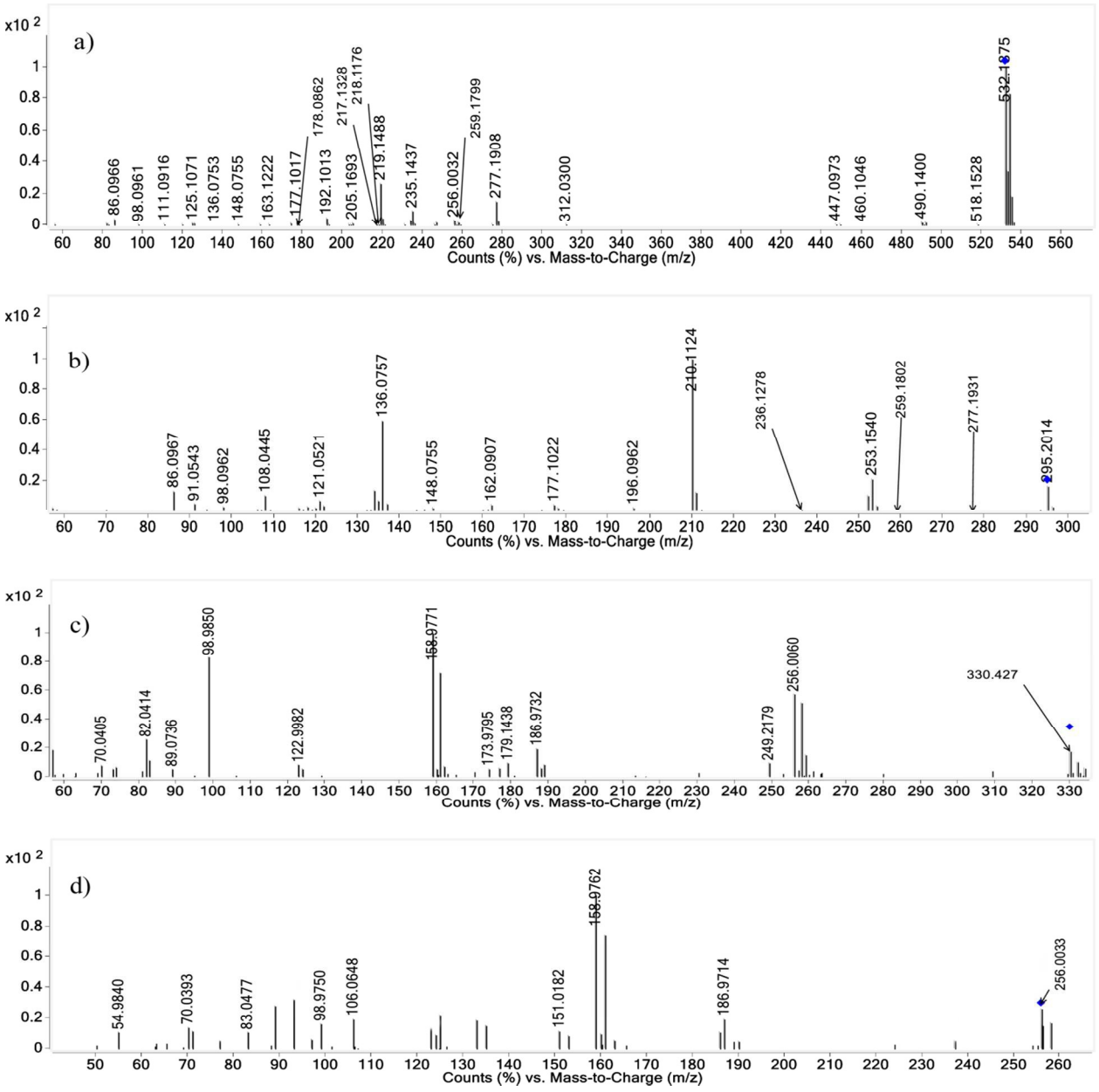

Fig. 1 UPLC-ESI/MS/MS spectrum of $[M + H]^+$ ions of (a) TER (*m/z* 532) at 25 eV, (b) DP1 (*m/z* 295) at 25 eV, (c) DP2 (*m/z* 330) at 25 eV, (d) DP3 (*m/z* 256) at 25 eV

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Table 1 High resolution mass spectrometry (HRMS) data of product ions of protonated terconazole (TER) and its degradation products

	Molecular formula	Calculated *m/z*	Observed *m/z*	Error (ppm)
TER	$C_{26}H_{32}Cl_2N_5O_3^+$	532.1877	532.1875	-0.38
	$C_{23}H_{26}Cl_2N_5O_3^+$	490.1407	490.1400	-1.43
	$C_{21}H_{21}Cl_2N_4O_3^+$	447.0985	447.0973	-2.68
	$C_{13}H_{12}Cl_2N_3O_2^+$	312.0301	312.0300	-0.32
	$C_{16}H_{25}N_2O_2^+$	277.1911	277.1908	-1.08
	$C_{16}H_{23}N_2O^+$	259.1805	259.1799	-2.31
	$C_{10}H_8Cl_2N_3O^+$	256.0039	256.0032	-2.73
	$C_{14}H_{23}N_2O^+$	235.1441	235.1437	-1.7
	$C_{13}H_{19}N_2O^+$	219.1492	219.1488	-1.83
	$C_{13}H_{16}NO_2^+$	218.1176	218.1176	0.00
	$C_{13}H_{17}N_2O^+$	217.1335	217.1328	-3.22
	$C_{13}H_{21}N_2^+$	205.1699	205.1693	-2.92
	$C_{11}H_{14}NO_2^+$	192.1019	192.1013	-3.12
	$C_{10}H_{12}NO_2^+$	178.0863	178.0862	-0.56
	$C_{11}H_{17}N_2^+$	177.1022	177.1017	-2.82
	$C_{10}H_{15}N_2^+$	163.1230	163.1222	-4.90
	$C_9H_{10}NO^+$	148.0757	148.0755	-1.35
	$C_8H_{10}NO^+$	136.0757	136.0753	-2.94
	$C_6H_{11}N2^+$	111.0917	111.0916	-0.90
	$C_5H_{12}N^+$	86.0964	86.0966	2.32
DP1	$C_{16}H_{27}N_2O_3^+$	295.2016	295.2014	-0.68
	$C_{16}H_{25}N_2O_2^+$	277.1911	277.1931	7.22
	$C_{16}H_{23}N_2O^+$	259.1805	259.1802	-1.16
	$C_{13}H_{21}N_2O_3^+$	253.1547	253.154	-2.77
	$C_{13}H_{18}NO_3^+$	236.1281	236.1278	-1.27
	$C_{11}H_{16}NO_3^+$	210.1125	210.1124	-0.48
	$C_{10}H_{14}NO_3^+$	196.0968	196.0962	-3.06
	$C_{10}H_{13}N_2O^+$	177.1022	177.1022	0.00
	$C_{10}H_{12}NO^+$	162.0913	162.0907	-3.70
	$C_9H_{10}NO^+$	148.0757	148.0755	-1.35
	$C_8H_{10}NO^+$	136.0757	136.0757	0.00
	$C_5H_{12}N^+$	86.0964	86.0967	3.48
DP2	$C_{13}H_{14}Cl_2N_3O_3^+$	330.0407	330.0404	-2.34
	$C_{10}H_8Cl_2N_3O^+$	256.0039	256.0036	-1.17
	$C_8H_5Cl_2O^+$	186.9712	186.9704	-4.28
	$C_7H_5Cl_2^+$	158.9763	158.9759	-2.52

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	$C_3H_4N_3^+$	82.0400	82.0398	-2.44
DP3	$C_{10}H_8Cl_2N_3O^+$	256.0039	256.0034	-1.95
	$C_8H_5Cl_2O^+$	186.9712	186.9713	0.53
	$C_7H_5Cl_2^+$	158.9763	158.9762	-0.63
	$C_3H_4N_3^+$	83.0491	83.0486	-6.02
DP4	$C_{24}H_{30}Cl_2N_5O_3^+$	506.1720	506.1715	-0.99
	$C_{21}H_{21}Cl_2N_4O_3^+$	447.0985	447.0977	-1.79
	$C_{19}H_{19}Cl_2N_4O_3^+$	421.0829	421.0817	-2.85
	$C_{13}H_{12}Cl_2N_3O_2^+$	312.0301	312.0291	-3.20
	$C_{10}H_8Cl_2N_3O^+$	256.0039	256.0032	-2.73
	$C_{11}H_{14}NO_2^+$	192.1019	192.1010	-4.69
	$C_{11}H_{12}NO^+$	174.0913	174.0905	-4.60
	$C_{10}H_{12}NO^+$	162.0913	162.0902	-6.79
	$C_9H_{10}NO^+$	148.0757	148.0750	-4.73
	$C_8H_8NO^+$	134.0600	134.0596	-2.98
	$C_7H_8NO^+$	122.0600	122.0593	-5.73
	$C_5H_{12}N^+$	86.0964	86.0963	-1.16
DP5	$C_{26}H_{32}Cl_2N_5O_5^+$	564.1775	564.1738	-6.56
	$C_{26}H_{31}Cl_2N_5O_4^{\bullet+}$	547.1748	547.1744	-0.73
	$C_{26}H_{30}Cl_2N_5O_4^+$	546.1669	546.1659	-1.83
	$C_{26}H_{28}Cl_2N_5O_3^+$	528.1564	528.1552	-2.27
	$C_{23}H_{26}Cl_2N_5O_5^+$	522.1306	522.1308	0.38
	$C_{23}H_{25}Cl_2N_5O_4^+$	505.1278	505.1263	-2.97
	$C_{23}H_{21}Cl_2N_5O_3^{\bullet+}$	485.1016	485.1012	-0.82
	$C_{21}H_{20}Cl_2N_4O_3^{\bullet+}$	446.0907	446.0900	-1.57
	$C_{13}H_{12}Cl_2N_3O_2^+$	312.0301	312.0304	0.96
	$C_{10}H_8Cl_2N_3O^+$	256.0039	256.0031	-3.12
	$C_6H_{12}N^+$	98.0964	98.096	-4.08
	$C_5H_{12}N^+$	86.0964	86.0966	2.32
DP6	$C_{26}H_{32}Cl_2N_5O_4^+$	548.1826	548.1825	-0.18
	$C_{26}H_{31}Cl_2N_5O_3^{\bullet+}$	531.1798	531.1790	-1.51
	$C_{26}H_{30}Cl_2N_5O_3^+$	530.1720	530.1713	-1.32
	$C_{25}H_{28}Cl_2N_5O_3^+$	516.1564	516.1568	0.77
	$C_{23}H_{24}Cl_2N_5O_3^+$	488.1251	488.1234	-3.48
	$C_{23}H_{23}Cl_2N_5O_3^{\bullet+}$	487.1172	487.1169	-0.62
	$C_{22}H_{23}Cl_2N_4O_3^+$	461.1142	461.1132	-2.17
	$C_{21}H_{20}Cl_2N_4O_3^{\bullet+}$	446.0907	446.0902	-1.12
	$C_{13}H_{12}Cl_2N_3O_2^+$	312.0301	312.0297	-1.28
	$C_5H_6N_3^+$	108.0556	108.0552	-3.70

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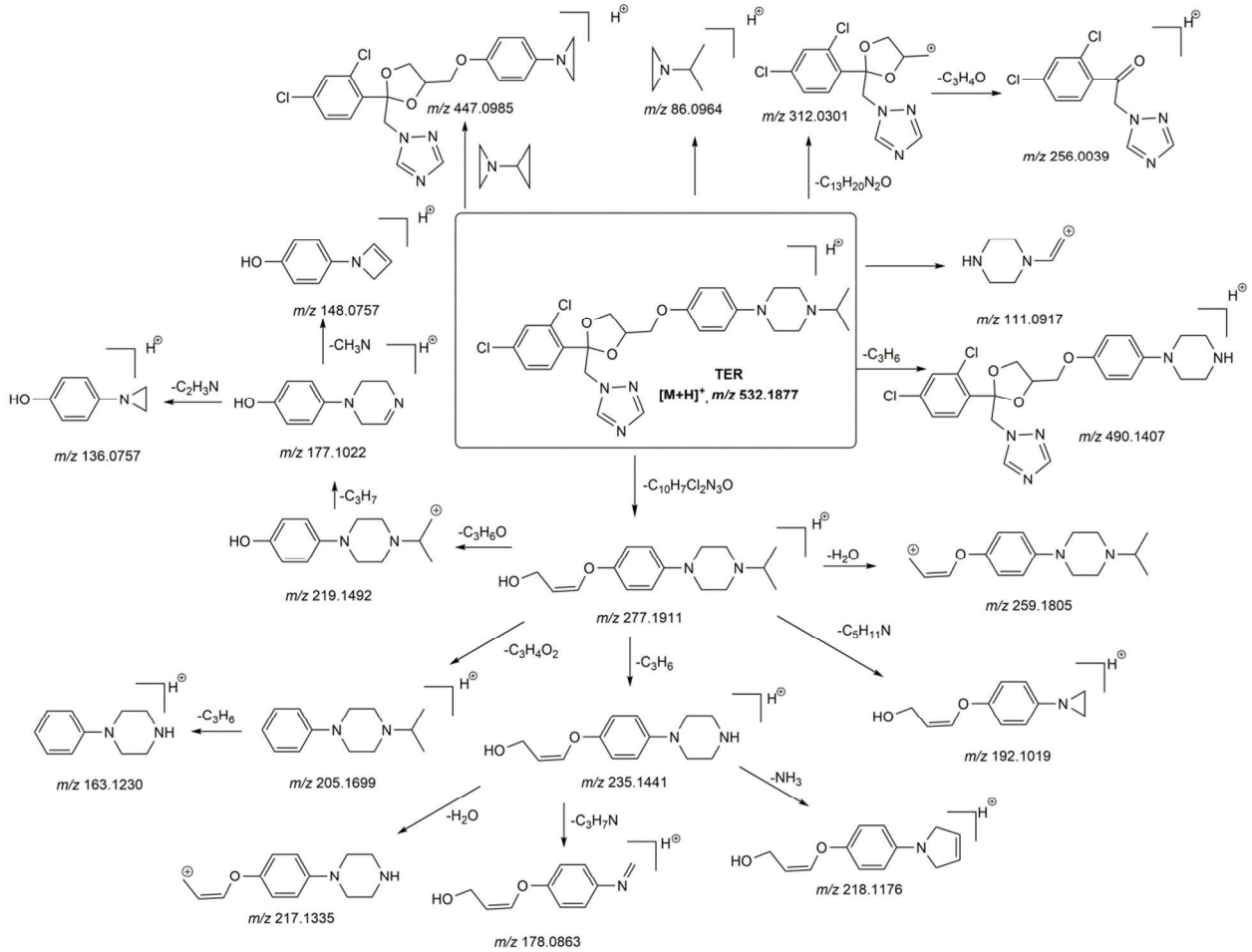
	$C_6H_{12}N^+$	98.0964	98.0960	-4.08
DP7	$C_{26}H_{32}Cl_2N_5O_4^+$	548.1826	548.1821	-0.91
	$C_{26}H_{31}Cl_2N_5O_3^{\bullet+}$	531.1798	531.1787	-2.07
	$C_{26}H_{30}Cl_2N_5O_3^+$	530.1720	530.1720	0.00
	$C_{25}H_{28}Cl_2N_5O_3^+$	516.1564	516.1562	-0.39
	$C_{23}H_{24}Cl_2N_5O_3^+$	488.1251	488.1233	-3.69
	$C_{23}H_{23}Cl_2N_5O_3^{\bullet+}$	487.1172	487.1165	-1.44
	$C_{22}H_{23}Cl_2N_4O_3^+$	461.1142	461.1128	-3.04
	$C_{21}H_{20}Cl_2N_4O_3^{\bullet+}$	446.0907	446.0904	-0.67
	$C_{13}H_{12}Cl_2N_3O_2^+$	312.0301	312.0298	-0.96
	$C_5H_6N_3^+$	108.0556	108.0556	0.00
	$C_6H_{12}N^+$	98.0964	98.0966	2.04



Scheme 1 Proposed ESI/MS/MS fragmentation pathway of protonated terconazole (TER)

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MS/MS of DPs

MS/MS of DP1 [$(M+H)^+$, *m/z* 295]

The degradation product DP1 was eluted at retention time (Rt) of 1.6 min. in acidic condition with molecular formula $C_{16}H_{27}N_2O_3^+$. The ESI/MS/MS spectrum **(Fig. 1b)** of $[M+H]^+$ ion (*m/z* 295) showed abundant product ions at *m/z* 210 and *m/z* 136 formed by the loss of C_2H_5N from *m/z* 253 and loss of C_2H_2 from *m/z* 162, respectively. Other product ions include *m/z* 277, *m/z* 259, *m/z* 253, *m/z* 236, *m/z* 196, *m/z* 177, *m/z* 162, *m/z* 148, and *m/z* 86 **(Scheme 2)**. The presence of two nitrogen atoms in the molecular formula ($C_{16}H_{27}N_2O_3^+$) and formation of characteristic fragment ion at *m/z* 86, indicates that the DP 1 was formed by the hydrolytic cleavage of carbon oxygen bonds by removal of 1-(2,4-dichlorophenethyl)-1H-1,2,4-triazole moiety. The consecutive water loss to form fragment ions at *m/z* 277 and *m/z* 259 from DP1 (*m/z* 295) and *m/z* 277, respectively confirms the presence of two aliphatic alcohol moieties in the structure of DP1. Absence of characteristic isotopic pattern of two chlorine (ratio of 9:6:1) in the molecular ion of DP1 indicates that dichlorobenzene moiety is removed during hydrolysis. Further the presence of structure indicative fragment ions at *m/z* 253, *m/z* 236, *m/z* 210 and *m/z* 196, confirm the DP 1 was 3-(4-(4-isopropylpiperazin-1-yl)phenoxy)propane-1,2-diol. A probable mechanism for the formation of DP1 is given in **Scheme S1**.

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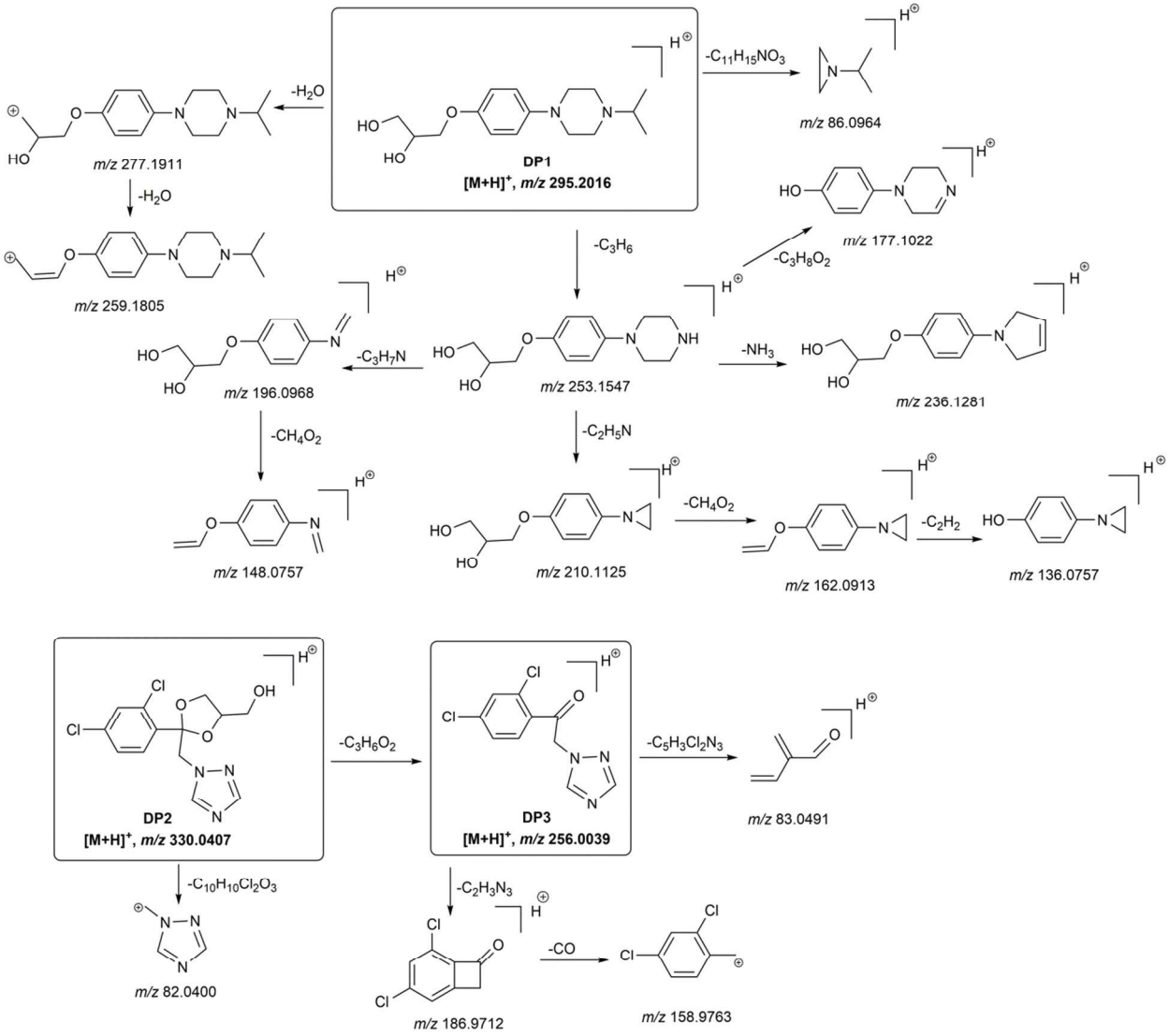

Scheme 2 Proposed ESI/MS/MS fragmentation pathway of protonated degradation product DP1, DP2 and DP3

MS/MS of DP2 [(M+H)$^+$, *m/z* 330]

The DP2 was observed in acidic and oxidative condition and eluted at Rt of 6.0 min. with its $[M+H]^+$ peak at *m/z* 330 **(Fig. 1c).** The MS/MS spectrum of DP2 shows highly abundant product ions at *m/z* 158 formed by decarbonylation (CO) from *m/z* 186 and *m/z* 256 formed by the loss of prop-1-ene-1,2-diol from *m/z* 330. Other product ions include *m/z* 186, *m/z* 82 (**Scheme 2).** The presence of characteristic product ion peak at *m/z* 82 (due to methyl triazole) and *m/z* 158 (due to

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methyl dichlorobenzene) proved that the structure of DP2 was (2-((1H-1,2,4-triazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)methanol. The formation of DP1 and DP2 involves the acid catalyzed nucleophilic substitution reaction. A probable mechanism for the formation of DP2 is given in **Scheme S2**.[13]

MS/MS of DP3 [(M+H)$^+$, *m/z* 256]

The acidic degradation product DP3 was eluted at Rt of 6.2 min. with its $[M+H]^+$ peak at *m/z* 256 **(Fig. 1d).** The MS/MS spectrum shows product ions at *m/z* 186 (loss of $C_2H_3N_3$ from *m/z* 256), *m/z* 158 (loss of CO from *m/z* 186), *m/z* 83 (loss of $C_5H_3Cl_2N_3$ from *m/z* 256) (**Scheme 2).** The absence of characteristic product ions peak at *m/z* 86 (due to isopropylaziridine was present in MS/MS of the drug) indicates that piperazine moiety was removed from the drug during hydrolysis. Probable mechanism for the formation of DP3 is provided in **Scheme S1**.

MS/MS of DP4 [(M+H)$^+$, *m/z* 506]

Figure 2a displays the ESI/MS/MS spectrum of DP4 which shows $[M+H]^+$ at *m/z* 506 with elemental composition $C_{24}H_{30}Cl_2N_5O_3^+$, two carbon atoms less than the TER ($C_{26}H_{32}Cl_2N_5O_3^+$) which indicates the elimination of two carbon atoms which in turn indicates that DP4 could be formed by the hydrolytic cleavage of the piperazine ring which leads to elimination of ethene moiety. MS/MS spectrum of DP4 displays the structure indicative fragment ions at *m/z* 447 and *m/z* 421 formed by the loss of propan-2-amine and -$C_5H_{11}N$ from *m/z* 506, respectively. Other fragment ions include *m/z* 312, *m/z* 256, *m/z* 192, *m/z* 174, *m/z* 162, *m/z* 148, *m/z* 134, *m/z* 122 and *m/z* 86 (**Scheme 3).** All the fragment ions and molecular formula were consistent that DP4 was N1-(4-((2-((1H-1,2,4-triazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolayl)methoxy)phenyl)-N2-isopropylethane-1,2-diamine. A probable mechanism for the formation of DP4 is shown in **Scheme S3**.

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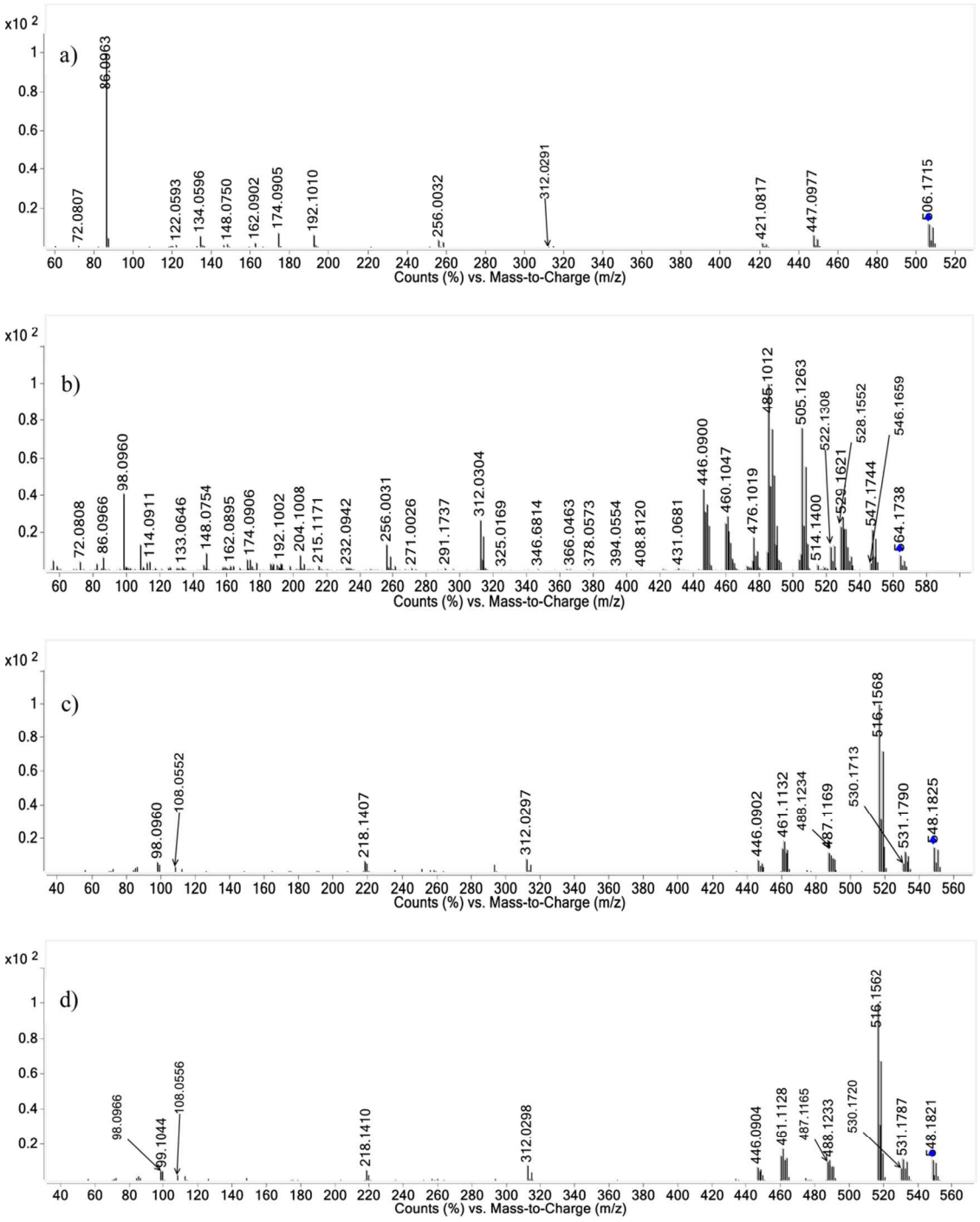

Fig. 2 UPLC-ESI/MS/MS spectrum of $[M + H]^+$ ions of (a) DP4 (*m/z* 506) at 30 eV, (b) DP5 (*m/z* 564) at 25 eV, (c) DP6 (*m/z* 548) at 25 eV and (d) DP7 (*m/z* 548) at 25 eV

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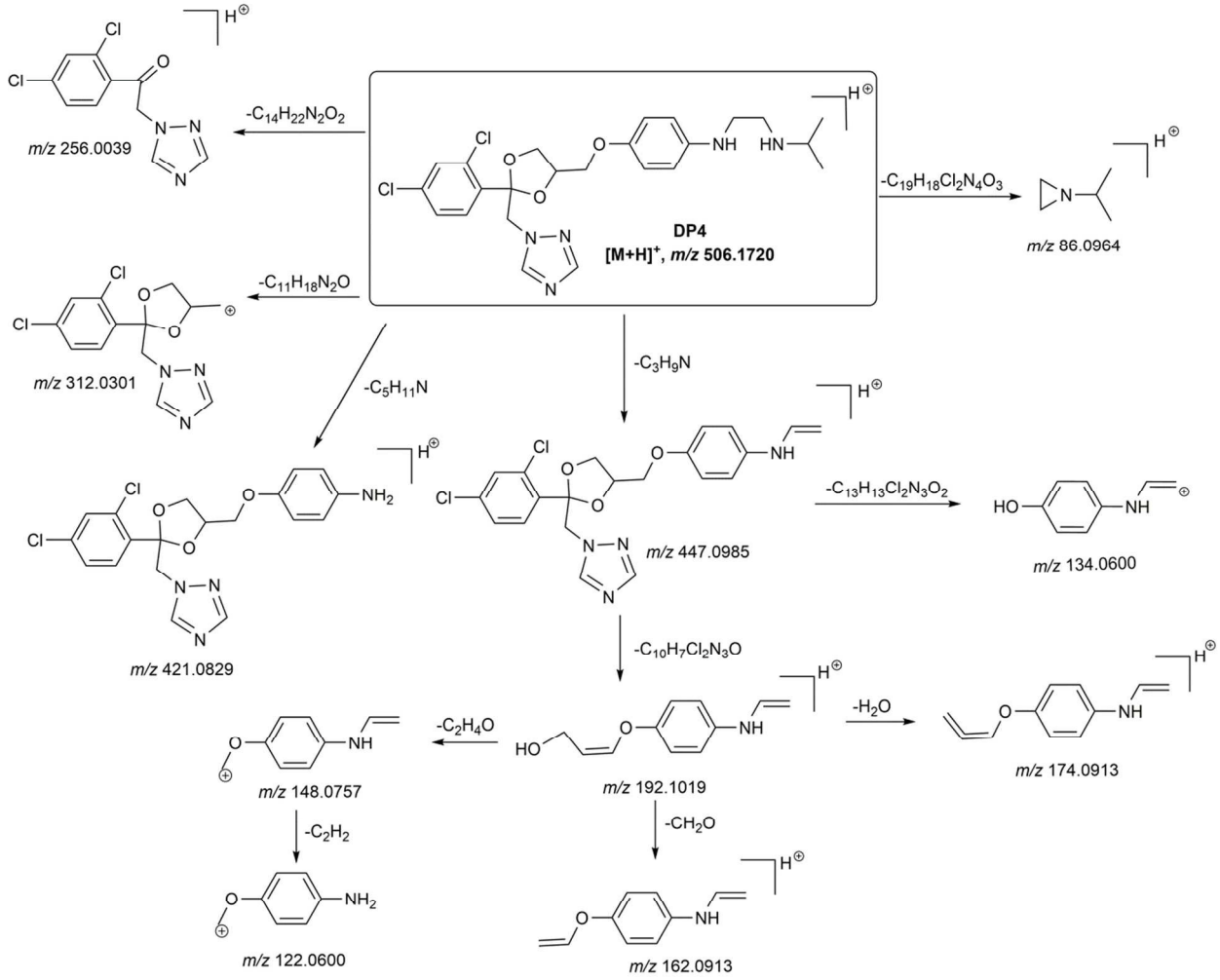

Scheme 3 Proposed ESI/MS/MS fragmentation pathway of protonated degradation product DP4

MS/MS of DP5 [(M+H)$^+$, *m/z* 564]

The **Figure 2b** shows the ESI-MS/MS spectrum of DP5 [M+H]$^+$ ion peak at *m/z* at 564 (Rt = 5.4 min). The mass difference between DP5 (*m/z* 564) and protonated drug (*m/z* 532) is 32 Da, indicates DP5 was formed by the addition of two oxygen atoms in the drug moiety. The mass spectrum of [M+H]$^+$ DP5 display the product ions at *m/z* 547 (loss of OH from *m/z* 564), *m/z* 546 (loss of H_2O from *m/z* 564), *m/z* 528 (loss of $2H_2O$ from *m/z* 564), *m/z* 522 (loss of C_3H_6 from *m/z* 564), *m/z* 505 (loss of C_3H_6 from *m/z* 547), *m/z* 485 (loss of C_3H_7 from *m/z* 528), *m/z* 446 (loss of C_2HN from *m/z* 485), *m/z* 312 (loss of $C_{13}H_{16}N_2O$ from *m/z* 528), *m/z* 256 (loss of

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C_3H_4O from *m/z* 312), *m/z* 98 and *m/z* 86 **(Scheme 4).** The MS/MS spectrum shows instantaneous loss of OH radical leads to formation of $[MH\text{-}OH]^+$ ion (*m/z* 547) which indicate the possibility of N-oxide degradation product [14]. The fragment ions at *m/z* 546 and *m/z* 528 could be formed by consecutive loss of water from the DP5. The formation of characteristic product ion of *m/z* 522 indicates the oxidation of the piperazine moiety. The formation of daughter ions at *m/z* 522 can be explained by the meisenheimer rearrangement followed by loss of propene. It can be observed that N-oxide instantly loses the OH radical giving rise to $[MH\text{-}OH]^{+\bullet}$ ions (*m/z* 547) in the MS/MS spectrum of *m/z* DP5.[15] Based on the above data DP5 could be N, N-dioxide of the TER. To confirm the formation of N, N-dioxide ^1H and ^{13}C NMR experiments were carried out. The data from NMR experiments (^1H and ^{13}C NMR) revealed that the difference between TER and DP5 are specific to the isopropyl group and piperazine ring (**Fig. 3**). The number of proton signals in the ^1H NMR TER (**Fig. S3**) and ^1H NMR of DP5 are similar which excludes the possibility of hydroxylation and confirm that DP5 was formed by N, N-dioxidation. The ^1H NMR signals of DP5 (**Fig. S4**) for protons at H1/H3, H5/H9, and H6/H8 was shifted to downfield region as compared to ^1H NMR signals of the drug (**Table 2**). Furthermore, compared to the ^{13}C NMR signals of TER (**Fig. S5**) the ^{13}C signals of DP5 assigned as C5/C9 and C6/C8 are significantly deshielded (**Fig. S6 and Table 3**). Based on above data the structure of the DP5 was characterized as 1-(4-((2-((1H-1,2,4-triazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)methoxy)phenyl)-4-isopropylpiperazine 1,4-dioxide. The probable mechanism for formation of DP5 is shown in **Scheme S4.**

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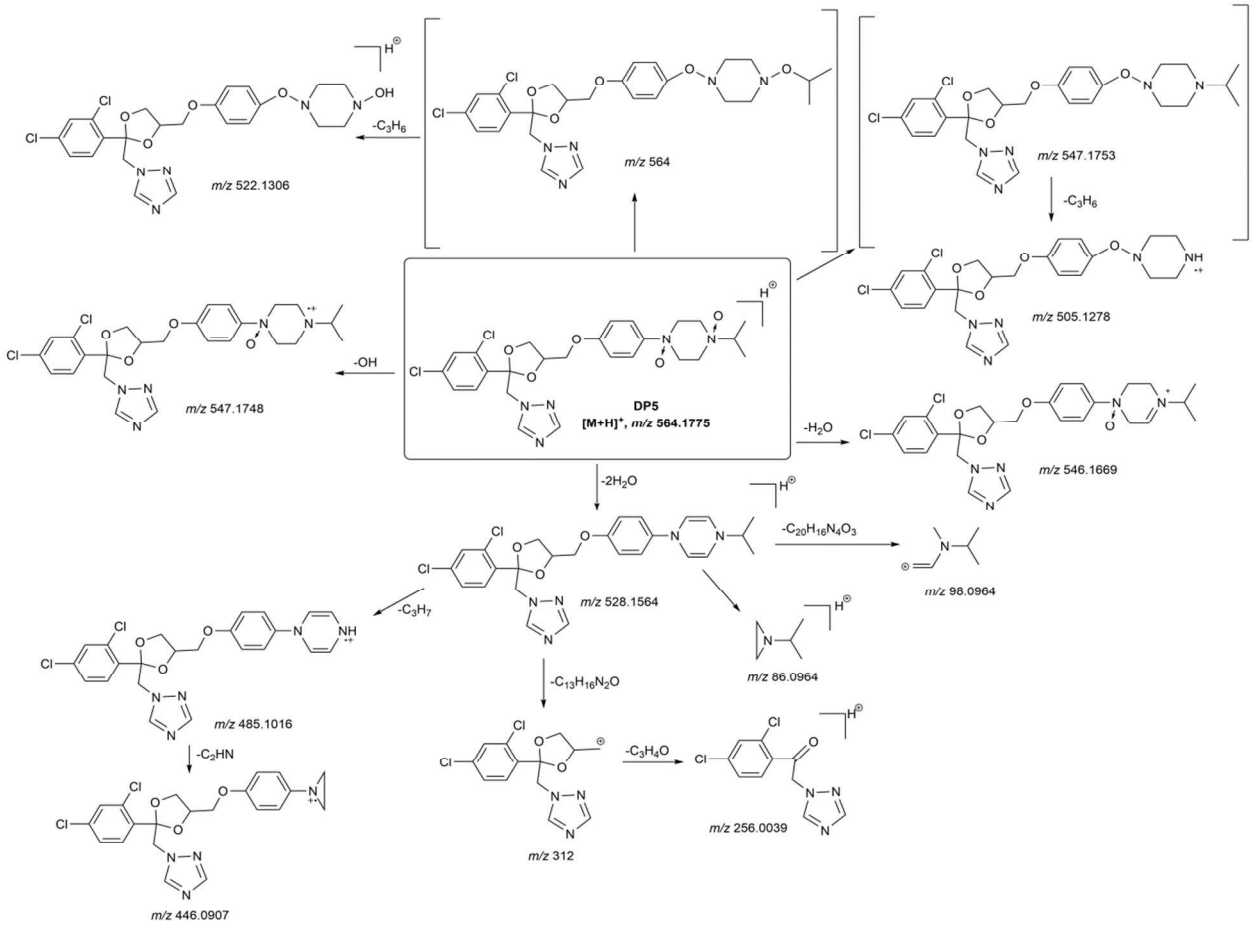

Scheme 4 Proposed ESI/MS/MS fragmentation pathway of protonated degradation product DP5

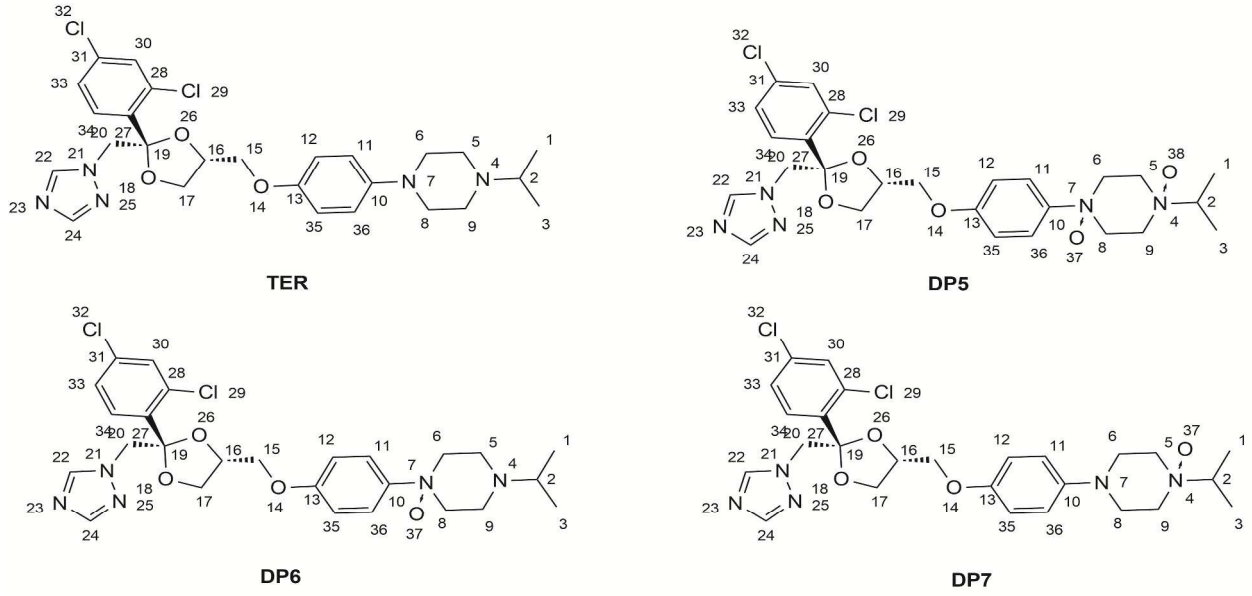

Fig. 3 Structures of TER, DP5, DP6 and DP7 with numbering for NMR values

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Table 2 ^{1}H NMR assignments for terconazole and DPs

Position[a]	Terconazole	DP5	DP6	DP7
	δ_H (ppm), Multiplicity	δ_H (ppm), Multiplicity	δ_H (ppm), Multiplicity	δ_H (ppm), Multiplicity
1/3	1.15 (d, 6H)	1.51 (d, 6H)	1.21 (d, 6H)	1.47 (d, 6H)
2	4.35 (m, 1H)	4.39 (m, 1H)	4.38 (m, 1H)	4.36 (m, 1H)
5a/9a 5e/9e	2.78 (t, 4H)	3.45 (t, 4H)	2.92 (d, 2H) 3.52 (d, 2H)	3.33 (d, 2H) 3.41 (td, 2H)
6a/8a	3.18 (t, 4H)	3.81 (m, 4H)	3.82 (m, 2H)	3.67 (t, 4H)
6b/8b	-	-	3.55 (m, 2H)	-
11/36	6.77 (d, 2H)	6.94 (d, 2H)	6.92 (d, 2H)	6.79 (d, 2H)
12/35	6.89 (d, 2H)	7.27 (d, 2H)	7.27 (d, 2H)	6.92 (d, 2H)
15a	2.81 (m, 1H)	3.56 (dd, 1H)	2.98 (dd, 1H)	3.89 (dd, 1H)
15b	3.49 (dt, 1H)	3.94 (dd, 1H)	3.3 (s, 1H)	3.51 (dd, 1H)
16	3.91 (dd, 1H)	4.89 (t, 1H)	3.93 (dd, 1H)	3.92 (dd, 1H)
17	3.8 (dt, 2H)	4.49 (m, 2H)	4.11 (s, 2H)	3.8 (dt, 2H)
20a	4.8 (dd, 2H)	4.81 (m, 2H)	4.8 (dd, 2H)	4.8 (dd, 2H)
22	8.2 (s, 1H)	8.22 (s, 1H)	8.2 (s, 1H)	8.2 (s, 1H)
24	7.89 (s, 1H)	7.91 (s, 1H)	7.91 (s, 1H)	7.89 (s, 1H)
30	7.47 (d, 1H)	7.58 (d, 1H)	7.59 (d, 1H)	7.47 (d, 1H)
33	7.25 (dd, 1H)	7.48 (dd, 1H)	7.48 (dd, 1H)	7.25 (dd, 1H)
34	7.56 (d, 1H)	7.96 (d, 1H)	7.90 (d, 1H)	7.57 (d, 1H)

S= singlet; d= doublet; t= triplet; m= multiplet; dd= doublet of doublets; dt= doublet of triplet
[a] numbering as given in Fig. 3

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Table 3 ^{13}C NMR assignments for terconazole and DPs

Position[a]	Terconazole	DP5	DP6	DP7
	δ_C (ppm)	δ_C (ppm)	δ_C (ppm)	δ_C (ppm)
1/3	18.40	16.42	18.18	16.57
2	54.94	55.45	55.10	45.39
5/9	48.70	62.46	43.44	70.57
6/8	50.41	70.79	66.68	58.85
10	146.13	145.37	146.15	144.92
11/36	115.23	115.08	114.92	115.36
12/35	118.24	121.75	121.83	119.03
13	152.37	158.78	158.53	153.24
15	67.67	67.18	67.18	67.38
16	74.73	74.35	74.37	74.68
17	67.47	67.46	67.39	67.60
19	107.62	107.78	107.77	107.62
20	53.64	53.53	53.54	53.63
22	144.90	144.95	144.95	144.59
24	151.39	151.29	151.30	151.23
27	134.09	133.84	133.85	133.14
28	133.14	133.14	133.15	134.01
30	131.43	131.51	131.52	131.45
31	136.05	136.20	136.21	136.10
33	127.25	127.32	127.33	127.27
34	129.63	129.60	129.61	129.62

[a] numbering as given in Fig. 3

MS/MS of DP6 and DP7 [(M+H)$^+$, *m/z* 548] (Isomeric *N-oxides*)

The ESI/MS/MS spectra of DP6 (Rt = 6.4 min) and DP7 (Rt = 6.8 min) shows the $[M+H]^+$ ion peak at *m/z* 548, formed under oxidation condition. A mass difference of 16 Da between protonated drug (*m/z* 532) and both protonated DPs (DP6 and DP7) suggest the addition of oxygen in presence of hydrogen peroxide to TER. The formation of fragment ions at *m/z* 531 and *m/z* 530 with the loss of -OH and H_2O from *m/z* 548 respectively, indicating DP6 and DP7 could be the *N*-oxides of TER.[14] **Figure 2c** and **d** shows the ESI-MS/MS spectrum of DP6 and DP7.

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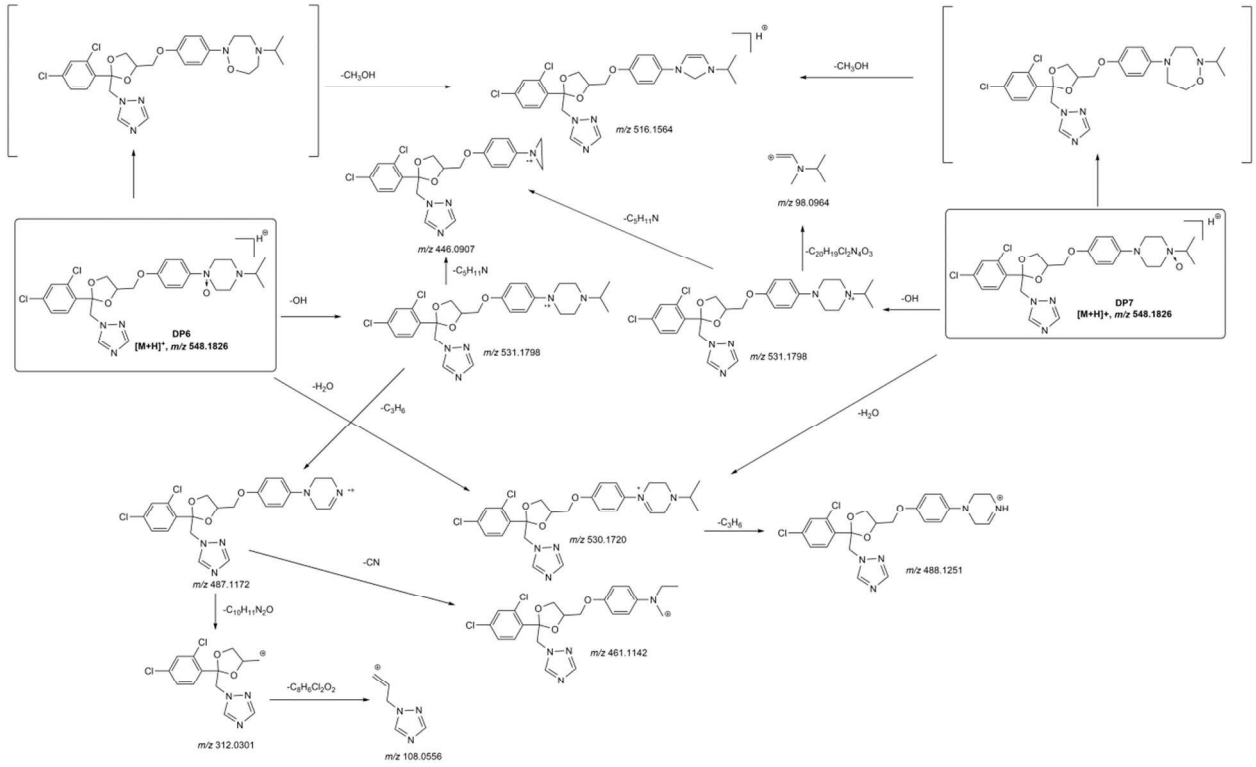
The ESI/MS/MS spectrum displays highly abundant product ion at *m/z* 516, which could be formed by the insertion of the oxygen into the piperazine ring of DP6 and DP7 to form an intermediate and subsequent loss of methanol from the intermediate.[15] The MS/MS spectra also display low abundance similar product ions at *m/z* 488, *m/z* 487, *m/z* 461, *m/z* 446, *m/z* 312, *m/z* 108 and *m/z* 98 **(Scheme 5).** To demonstrate the presence of *N*-oxides and to define the position of oxidation, APCI-MS experiments were conducted in scan mode. The formation of fragment ion at *m/z* 532 in full scan APCI-MS, considering thermally induced de-oxygenation in the vaporizer of the APCI source, confirms the formation of *N*-oxide.[16] Further APCI spectrum of DP7 also displays characteristic fragment ion at *m/z* 506 derived from Meisenheimer rearrangement, on the contrary DP6 did not show such characteristic ion **(Fig. 4a, b).**[17]



Scheme 5 Proposed ESI/MS/MS fragmentation pathway of protonated degradation product (DP6 and DP7)

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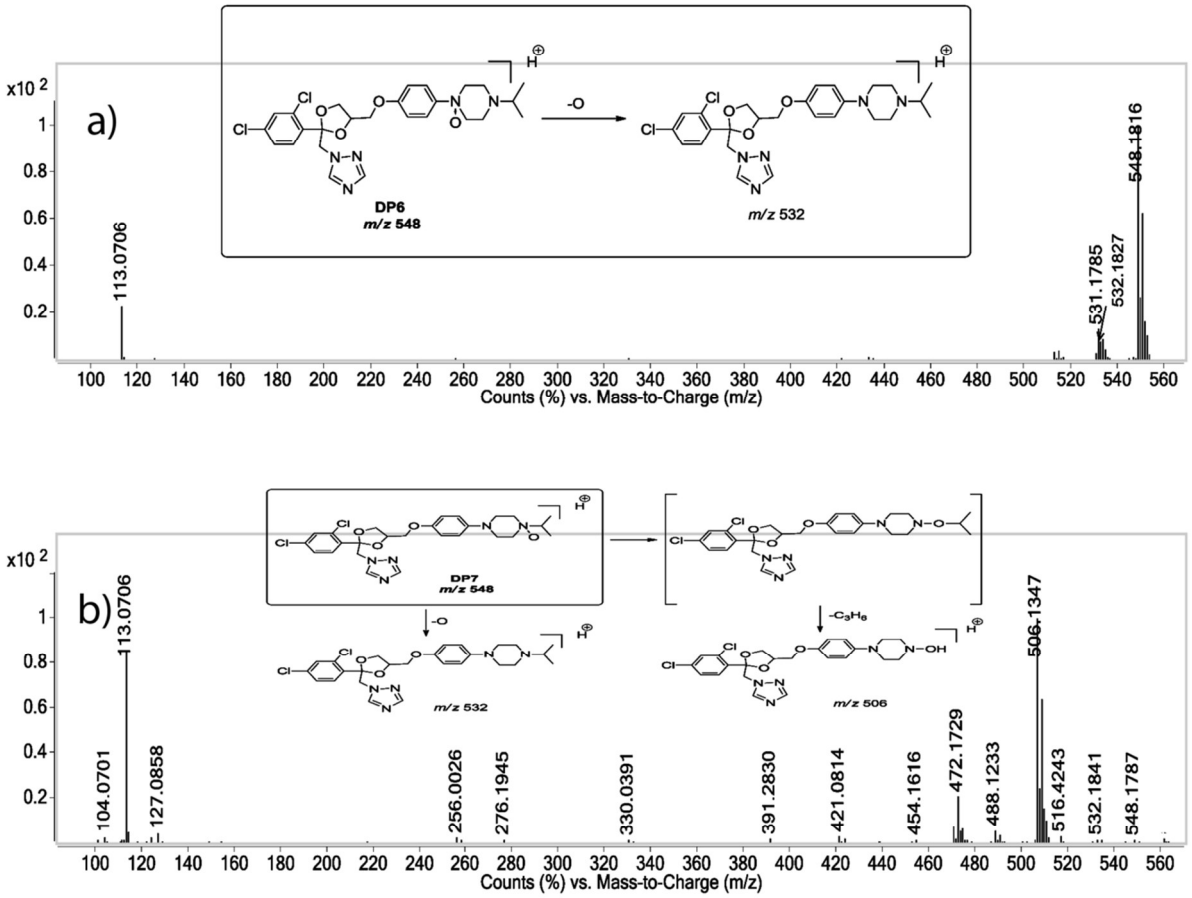

Fig. 4 Full scan UPLC-APCI/MS spectrum of (a) DP6 (*m/z* 548) and (b) DP7 (*m/z* 548)

However, to confirm the positions of *N*-oxides in DP6 and DP7 based on data provided by mass spectrometric experiments, NMR (^{1}H and ^{13}C NMR) experiments were performed. The possibility of hydroxylation can be ruled out as the numbers of proton signals are similar to that of drug. Based on the NMR data of both the DPs the oxidation was expected to be on piperazine ring of the TER as there were no notable differences in the NMR signals of any other moieties. According to the ^{1}H NMR signals of DP6 (**Fig. S7**) significant deshielding was observed for the protons at H5/H9 and H6/H8 whereas no change in the delta values of H1/H3 protons was noticed which concludes that DP6 was formed by the oxidation of the nitrogen atom attached to

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the phenyl ring (N7) **(Table 2).** The proton signals for the H5/H9 and H6/H8 were split into two signals. Additionally, ^{13}C spectrum of DP6 shows considerable downfield shift for the C6/C8 while C5/C9 which is β-carbons of N-7 has slightly shifted upfield (**Fig. S8 and Table 3**).

In case of DP7, the ^{1}H NMR spectrum shows significant downfield shift for the protons at H1/H3 and H5/H9 along with H6/H8 which confirms N-4 was oxidized to form DP7 (**Fig. S9**). The protons signals at H5/H9 were split into two resonances. The ^{13}C NMR spectrum of DP7 displays expected downfield shift for C5/C9 and C6/C8 as compared to the drug whereas shielding effect was observed for the C2 and C1/C3 (**Fig. S10 and Table 3**)**.** All this data proves that DP6 and DP7 are oxidative DPs and formed by the oxidation of nitrogen where phenyl (N7) and isopropyl group (N4) is attached, respectively. Based on above discussion, probable structures for DP6 and DP7 could be given as 1-(4-((2-((1H-1,2,4-triazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)methoxy)phenyl)-4-isopropylpiperazine 1-oxide, and 4-(4-((2-((1H-1,2,4-triazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4 yl)methoxy)phenyl)-1-isopropylpiperazine 1-oxide, respectively. The elemental composition of DPs and its product ions were confirmed by accurate mass measurement. A probable mechanism of formation of DP6 and DP7 is shown in **Scheme S4**.

Conclusion

A simple validated stability indicating UPLC method was developed for quantitative determination of TER and PIs in drug substance and drug product. In addition, stress degradation behavior of TER was studied according to ICH guidelines. Acid hydrolysis of TER yields four DPs (DP1, DP2, DP3, and DP4) and four DPs (DP2, DP5, DP6 and DP7) were formed in oxidative stress condition. The exposure of the drug to oxidative stress conditions resulted in the generation of isomeric N-oxides, DP6 and DP7. The present study shows the application of

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APCI technique in addition to elctrospray ionization (ESI) technique for the identification of N-oxide degradation products (DP6-DP7) formed under oxidative stress condition. ESI/MS/MS and full scan APCI/MS utilized to characterize the DPs formed under acid hydrolytic and oxidative stress conditions. In addition, structures of oxidative DPs (DP5, DP6 and DP7) were accurately established using ^{1}H NMR and ^{13}C NMR experiments. This method may be useful for the routine quality control analysis of drug and it is also suitable for detecting trace level of impurities and degradation products.

Conflicts of interest

There are no conflicts to declare.

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