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Characterization of stress degradation products of tolvaptan by UPLC-Q-TOF-MS/MS

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Abstract

Tolvaptan (TVT) is a selective, competitive vasopressin receptor 2 antagonist used to treat hyponatremia. TVT was subjected to forced degradation under the conditions of hydrolysis, oxidation, dry heat and photolysis, in accordance with the ICH guideline Q1A (R2) and degradation products (DPs) formed have been characterized through UPLC-PDA and UPLC-Q-TOF-MS/MS studies. The chromatographic separation was achieved on Acquity UPLC HSS T3 column (100 x 2.1 mm, 1.7 µm) with mobile phase containing a gradient mixture of solvent A (0.1% Formic acid) and B (Acetonitrile) at a flow rate of 0.3 ml/min at 30 °C. The detection wavelength was set at 266 nm. The drug degraded under acid hydrolysis, base hydrolysis and oxidative conditions to form a total of 7 DPs. When methanol was used as co-solvent during stress degradation, four additional degradation products were formed which were absent when acetonitrile was used as co-solvent. Comparison of the fragmentation pattern of DPs with that of the drug helped in the elucidation of structures of all the degradation products. The degradation pathway of the drug was established, which was duly justified by mechanistic explanation. The developed UPLC method was validated as per ICH guidelines.

Keywords: Tolvaptan; forced degradation; UPLC; LC-MS/MS; Stress testing; stability indicating method.

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1. Introduction

Tolvaptan (TVT) is chemically known as (±)-4'-[(7-chloro-2,3,4,5-tetrahydro-5-hydroxy-1*H*-1-benzazepin-1-yl) carbonyl]-o-tolu-m-toluidide, is an oral non peptide selective vasopressin V_2-receptor antagonist[1-4]. It is indicated for the treatment of clinically relevant hypervolemic or euvolemic hyponatremia associated with heart failure, cirrhosis, or syndrome of inappropriate antidiuretic hormone[1, 2]. TVT selectively inhibits arginine vasopressin (AVP) induced water reabsorption in the kidney by competitively blocking the binding of AVP to V_2-receptors in the distal nephron, thereby preventing the antidiures caused by circulating AVP [3-5].

Characterization of degradation products is an important task in drug discovery and development process.The international conference on harmonization (ICH) guidelines emphasizes to identify the likely degradation products, degradation pathways and intrinsic stability of the molecule. Hence, it is necessary to carry out the stability studies of drug under various forced degradation conditions of hydrolysis, oxidation, thermal and photolysis.

Analytical methods for estimation of TVT include estimation in biological samples[6-10] and in formulation[11]. Liquid chromatography–tandem mass spectrometry method for determining tolvaptan and its nine metabolites in rat serum has also been reported which involves gradient elution using 0.3 % acetic acid and acetonitrile with 0.3 % acetic acid on C18 column employing ESI positive ionization mode[12]. Stability methods by UPLC[13] and HPLC[14] for impurity profiling of TVT are reported in which however identification of degradation products have not been attempted. Govind Reddy et al have reported RP-HPLC method for estimation of process impurities in presence of degradation products with only three known DPs whereas other were not characterized[15]. In the present work, to understand better the mechanism of degradation of TVT and characterize the degradation products, a chromatographic method involving diode array

detector (DAD) and tandem mass spectrometry (MS/MS) is developed. Degradation of TVT according to the ICH guidelines was performed and mechanism for the TVT degradation has been established.

2. Experimental

2.1. Chemicals and Reagents

Pure tolvaptan was supplied by MSN Laboratories limited (Hyderabad, India). Commercially available 15 mg tolvaptan tablets (TOLVAT, Sun Pharmaceuticals, Mumbai, India) were purchased from local pharmacy. The HPLC grade acetonitrile (ACN), methanol (MeOH) and formic acid were purchased from Merck (Mumbai, India). Ammonium acetate (HPLC Buffer grade) and Hydrogen peroxide (30% w/v) were purchased from Finar Chemicals Pvt Ltd. (Ahmedabad). Analytical reagent (AR) grade sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Fine Chemicals Limited, Ahmedabad. High purity water was prepared by using Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

2.2. Instrumentation

The UPLC system used was Acquity UPLC H Class system from Waters (Waters, Milford, MA, USA) with quaternary solvent manager, autosampler, column oven and PDA detector. Data acquisition, analysis, and reporting were performed by Empower3 (Waters) chromatography software.

For LC-MS characterization, an Agilent 1290 series LC instrument (Agilent Technologies, USA) attached to a quadrupole – time of flight (Q-TOF) mass spectrometer (Q-TOF-LC/MS) 6540 series, Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source was used. Data analysis was carried out using Mass Hunter workstation software.

The typical operating source conditions were optimized as follows: the fragmentor voltage was set at 140 V; the capillary at 3500 V; the skimmer at 65 V; nitrogen was used as the drying (325°C; 10 L/min) and nebulizing (40 psi) gas. All the spectra were recorded under identical experimental conditions, and are an average of 25 scans. MS scan were carried out in positive ESI mode.

A photo stability chamber (Osworld) consisting of both UV and fluorescent lamp was used for the photo degradation study. A water bath equipped with temperature controller was used to carry out degradation studies for all solutions. A controlled temperature dry air oven (Osworld laboratory oven) was used for solid-state thermal stress study.

2.3. Chromatographic Conditions

The chromatographic separations were carried out on Acquity UPLC HSS T3 column (100 x 2.1 mm, 1.7 µm) with mobile phase containing a gradient mixture of solvent A (0.1% formic acid) and B (Acetonitrile) at a flow rate of 0.3 ml/min. The gradient programme was set as follows: (Time in min./% proportion of solvent B): 0-0.5/25, 0.50-2.50/45, 2.50-3.50/55, 3.50-5.50/60, 5.50-6.50/70, 6.50-7.00/25, 7.00-9.00/25. The eluted compounds were monitored at 266 nm. The column oven and auto sampler temperatures were maintained at 30 °C and 5 °C, respectively. An injection volume of 1µl was used.

2.4. Preparation of Stock and Standard Solutions

A standard stock solution of TVT (500 µg/ml) was prepared in HPLC grade ACN. Aliquots of the stock solution were transferred and diluted with ACN: water (50:50 v/v) to yield concentrations equal to 25, 50, 75, 100, 125 and 150 µg/ml. For assay of tablets, 20 tablets were crushed. After mixing the amount equivalent to one tablet was weighed and taken in 100 mL volumetric flask, dissolved in diluent (ACN: water (50:50 % v/v)), sonicated for 15 min, diluted

up to the mark with same solvent and filtered through a 0.45 mm whatmann filter paper. From this solution further dilutions were made with diluent to obtain the solution containing 100 µg/ml. This solution was analyzed for assay.

2.5. Forced degradation studies

2.5.1. Hydrolysis

For acid hydrolysis, 5 mg drug was dissolved in 1 mL of ACN and 4 mL of 1N HCl was added and kept for refluxing the solution at 80 °C for 24 hrs. The studies in alkaline conditions were done by dissolving 5 mg drug in 1 mL of ACN followed by addition of 4 mL of 2N NaOH and the solution was refluxed at 80 °C for 17hr. For neutral hydrolysis, drug was initially dissolved in ACN, diluted with water and the solution was refluxed at 80 °C for 48hr.

After completion of the degradation treatments, the samples were allowed to cool to room temperature, neutralized as needed and injected into the chromatographic system after appropriate dilution with the diluent (ACN: water-50: 50, v/v).

2.5.2. Oxidative degradation

TVT was dissolved in 1 mL of ACN and treated with solution of 10% (v/v) H_2O_2 at room temperature in the dark for 24 hrs.

2.5.3. Photolytic degradation

For solid sample, approximately 100 mg of TVT was spread on a petri-dish in a layer of 1 mm thickness. For solution, drug was initially dissolved in ACN, and then diluted with water. The samples were kept in a photo-stability chamber and exposed to 1.2 million lux hr of fluorescent light and 200 Wh/m^2 of illumination as per ICH conditions [8] for 3 days. A parallel set of the drug samples were stored in dark at the same temperature to serve as control.

2.5.4. Thermal degradation

The dry heat degradation was carried out by exposing the drug to a temperature of 80 °C for 7 days in a hot air oven.

2.6 Effect of co-solvent on degradation

Initially, ACN was used as co-solvent for all stress degradation studies. As MeOH is a cheaper solvent, it was also tried as co-solvent for sample preparation for stress degradation studies.

3. Results and Discussion

3.1. Method Development

In order to achieve the optimum resolution, numerous changes in the chromatographic conditions such as pH and composition of the mobile phase, flow rate, column, etc. were carried out. Different mobile phase compositions using buffers pH 3.0, pH 4.0, pH 5.5 and 0.1% formic acid with ACN and MeOH were tried. Isocratic trails were not successful in achieving a favorable resolution. The well resolved degradation product peaks along with a sharp and symmetrical drug peak were obtained using 0.1% formic acid in water and ACN using proposed gradient method with UPLC HSS T3 column. For LC/MS analysis, electrospray ionization (ESI) and atmospheric chemical ionization were tried in both positive and negative mode. It was observed that maximum intensity was obtained with ESI in positive mode. Mass spectrometer conditions were optimized to obtain maximum ionization of TVT and all the DPs. Experiments were conducted using different capillary voltage (3000 V, 3500 V and 4000 V) and different fragmentor voltage (100 V, 125 V, 140 V, 170 V) at constant source temperature of 325 °C. The maximum intensity of the analytes were observed using capillary voltage and fragmentor voltage at 3500 V and 140 V, respectively.

3.2. Degradation behavior of TVT

The optimized LC–MS method was used to identify the degradation products of TVT. The overlay of chromatograms of stress degradation samples are given in fig 1. A total of eleven DPs were identified and characterized by using LC/ESI/MS/MS experiments and accurate mass measurements. The proposed structures of DPs and their elemental compositions are given in Scheme 1 and Table 1.

3.2.1 Hydrolysis

The drug degraded significantly to form DP-1, DP-2, DP-3 DP-4, DP-8, DP-9 and DP-11 in acid hydrolysis. In alkaline hydrolysis conditions, TVT degraded to yield DP-1, DP-2, DP-4 and DP-5 whereas it was stable even after refluxing it in neutral hydrolytic condition for 48 hrs.

3.2.2 Oxidative degradation

The drug was degraded to form minor degradation products DP-6, DP-7, DP-10.

3.2.3 Photolytic degradation

No degradation was observed in photo-degradation condition.

3.2.4 Thermal degradation

The exposure of the solid drug to 80 °C for 7 days did not result in significant decomposition which indicates that TVT is stable to dry heat.

3.3 Effect of co-solvent on degradation

As shown in the fig. 1, DP-1, DP-3 & DP-9 in acid hydrolysis, DP-1 in base hydrolysis and DP-6 in oxidative degradation were formed when MeOH was used as co-solvent. However, they were not formed when exposed to same stress conditions using ACN as co-solvent. This suggested that DP-1, DP-3, DP-6 and DP-9 were formed due to presence of methanol and it is clear from the chemical structures of aforementioned degradation products.

3.4 UPLC–MS/MS of TVT and its degradation products

3.4.1 MS/MS of TVT

To elucidate the degradation behavior of TVT (retention time (Rt) = 6.55min), ESI-MS/MS spectrum (Fig. 2a) of its $[M+H]^+$ion (m/z 449) was examined. The spectrum shows abundant product ions at *m/z* 431 (loss of H_2O), *m/z* 252 (loss of $C_{10}H_{12}ClNO$), *m/z* 206 (loss of $C_{15}H_{15}NO$ from *m/z* 431), *m/z* 180 (loss of CO from *m/z* 206), *m/z* 119 (2-methylbenzylidyne) oxonium), *m/z* 91 (loss of CO from *m/z* 119) (scheme 2). It can be noted that the product ions at m/z 206 and 180 are characteristic for 7-chloro-5-hydroxy-2,3,4,5-tetrahydro-1H-benzo[b]azepine-1-carbonyl skeleton of TVT while the fragment ions with *m/z* 252 and 119 are diagnostic for 3-methylphenyl-2-methylbenzamide skeleton in TVT. The elemental compositions of all these ions have been confirmed by accurate mass measurements.

3.4.2 MS/MS of degradation products

On line LC–ESI-MS/MS experiments were performed to characterize all the degradation products (DP-1 – DP-11)) formed under various stress conditions. Most probable structures have been proposed for all the degradation products based on the *m/z* values of their $[M+H]^+$ions and the MS/MS data in combination with elemental compositions derived from accurate mass measurements, as discussed below.

DP-1

The ESI-MS/MS spectrum of $[M+H]^+$ion (*m/z* 212) of DP-1 (Rt = 3.18 min), is given in Fig. 2b. The MS/MS spectrum shows structure indicative fragment ions as shown in scheme 3(a). The peak at *m/z* 180 is diagnostic for 7-chloro-2,3-dihydro-1H-benzo[b]azepine which further fragments to give *m/z* 144 (loss of HCl). As shown in scheme 4(a), the mass difference of 32 u between $[M+H]^+$ion for DP-1(*m/z* 212) and DP-11 (*m/z* 180) indicated addition of methanol to

DP-11. Based on all these data, DP-1 was identified as 7-chloro-4-methoxy-2,3,4,5-tetrahydro-1H-benzo[b]azepine.

DP-2

The ESI-MS/MS spectrum of $[M+H]^+$ion (*m/z* 331) of DP-2 (Rt =4.08 min) (Fig. 2c) with an elemental composition of $C_{18}H_{20}ClN_2O_2$ shows product ions which are given in Scheme 3(b). The characteristic product ions of DP-2 include *m/z* 313 (loss of water), *m/z* 206 (loss of C_7H_9N from *m/z* 313), *m/z*134 (loss of $C_{10}H_{10}ClN$). Based on all these data, 4-amino-2-methylphenyl)(7-chloro-5-hydroxy-2,3,4,5-tetrahydro-1H-benzo[b]azepin-1-yl)methanone structure can be proposed for DP-2.

DP-3

ESI-MS/MS spectrum of $[M+H]^+$ion (*m/z* 345) of DP-3 (Rt = 4.40 min) is shown in Fig.3a. The spectrum displays product ions at *m/z* 238 (loss of C_7H_9N), *m/z* 206 (loss of CH_3OH from *m/z* 238, ((7-chloro-2,3-dihydro-1H-benzo[b]azepin-1-yl) methylidyne) oxonium) which are compatible with the structure, (4-amino-2-methylphenyl)(7-chloro-4-methoxy-2,3,4,5-tetrahydro-1H-benzo[b]azepin-1-yl) methanone (scheme 3d).The mechanism of formation of DP-3 is shown in scheme 4(a). It can be noted that the mechanism may involve an addition of methanol to DP-8.

DP-4

The ESI-MS/MS spectrum (Fig. 3b)of $[M+H]^+$ion of DP-4 (Rt = 4.98 min.) with an elemental composition $C_{16}H_{16}NO3^+$ displays product ions which are compatible with the structure, 2-methyl-4-(2-methylbenzamido) benzoic acid. The characteristic fragments for DP-4 include *m/z* 226 (loss of CO_2) and *m/z* 178 (loss of C_7H_8). A probable mechanism for the formation of DP-4

under hydrolytic condition may involve hydrolytic cleavage of carbonyl attached to benzazepine moiety of TVT (scheme 4(a)).

DP-5

The mass difference between the drug (m/z 449) and DP-5 (m/z 431) is 18 Da which suggests that it is formed by loss of water molecule from the drug in base hydrolysis. The elemental compositions of $[M+H]^+$of DP-5 and its product ions (scheme 3c) have been confirmed by accurate mass measurements (Table 2). All these data are highly compatible with the proposed structure, N-(4-(7-chloro-2,3-dihydro-1H-benzo[b]azepine-1-carbonyl)-3-methylphenyl)-2-methylbenzamide. As shown in the scheme 4(a), DP-5 may be formed by base catalyzed elimination of water from TVT.

DP-6

Fig. 4a shows the ESI-MS/MS spectrum of $[M+H]^+$ions (*m/z* 445) of DP-6 (Rt =5.52min) with an elemental composition of $C_{27}H_{29}N_2O_4$. Absence of chlorine isotope pattern in the spectrum confirmed that lone chlorine atom was absent in the DP-6. As explained in scheme 3c, the spectrum shows the characteristic product ions with *m/z* 427 (loss of H_2O), *m/z* 202 (loss of $C_{15}H_{15}NO$ from *m/z* 427), *m/z* 176 (loss of CO from *m/z* 202) consistent with the structure, N-(4-(5-hydroxy-7-methoxy-2,3,4,5-tetrahydro-1H-benzo[b]azepine-1-carbonyl)-3-methylphenyl)-2-methylbenzamide. The probable mechanism of formation of DP-6 in oxidative degradation is shown in the scheme 4(b).

DP-7

The ESI-MS/MS spectrum of $[M+H]^+$ion (m/z 435) of DP-7 is given in Fig. 4b. The spectrum shows structure indicative product ions which are explained in scheme 3(c) and listed in Table 2.

For example, *m/z* 416 (loss of H_2O) and *m/z* 105 (benzylidyne oxonium ion) indicated loss of methylene from TVT (scheme 4(b)) to form DP-7.

DP-8

The degradant DP-8 at *m/z* 313 ($[M + H]^+$) was eluted at 6.24 min. Its MS/MS spectrum (Fig. 4c) displays product ions at *m/z* 206 (((7-chloro-2,3-dihydro-1H-benzo[b]azepin-1-yl) methylidyne) oxonium), *m/z* 180 (7-chloro-2,3-dihydro-1H-benzo[b]azepine), *m/z* 134 ((4-amino-2-methylbenzylidyne)oxonium) and *m/z* 107 (loss of CO from *m/z* 134) (scheme 3b). Absence of the product ion at *m/z* 119 and presence of *m/z* 134 suggested probable structure of DP-8 would be, (4-amino-2-methylphenyl) (7-chloro-2,3-dihydro-1H-benzo[b]azepin-1-yl) methanone.

DP-9

The ESI-MS/MS spectrum of $[M+H]^+$ion (*m/z* 284) of DP-9 (Rt = 6.93 min), with elemental composition of $C_{17}H_{18}NO_3$ is given in Fig 5. The characteristic fragments of DP-9 (scheme 3d) include *m/z* 192 (loss of C_7H_8), *m/z* 252 (loss of OCH_3) and *m/z* 164 (loss of CO from *m/z* 192). These data indicated it to be, methyl 2-methyl-4-(2-methylbenzamido) benzoate. DP-9 may be formed by an esterification of DP-3 due to the presence of methanol in acid hydrolytic conditions (scheme 4a).

DP-10

DP-10 was formed in oxidative degradation condition through oxidation of alcohol to ketone. The ESI-MS/MS spectrum of $[M + H]^+$ ion of DP-10 (*m/z* 447, $C_{26}H_{24}NClN_2O_3$, Rt = 7.40 min) displays product ion at *m/z* 222 (((7-chloro-5-oxo-2,3,4,5-tetrahydro-1H-benzo[b]azepin-1-yl) methylidyne) oxonium ion). Presence of identical fragments of *m/z* 252 and *m/z* 119 in parallel to those of the drug indicated that oxidation occurred at N-(4-(7-chloro-5-hydroxy-2,3,4,5-tetrahydro-1H-benzo[b]azepine part of the drug. The mechanism of formation of DP-10 is

depicted in scheme 4(b). Accordingly, the structure of DP-10 may be, N-(4-(7-chloro-5-oxo-2,3,4,5-tetrahydro-1H-benzo[b]azepine-1-carbonyl)-3-methylphenyl)-2-methylbenzamide.

DP-11

The ESI-MS/MS spectrum (Fig. 5c) of $[M + H]^+$ ion of DP-11 (*m/z* 180, Rt = 7.45 min) displays product ions that are compatible with the structure, 7-chloro-2,3-dihydro-1H-benzo[b]azepine. The elemental compositions of DP-11 and its product ions have been confirmed by accurate mass measurements (Table 2). A probable mechanism for the formation of DP-11 under acid hydrolysis conditions may involve hydrolytic cleavage of amide functionality in DP-8 (scheme 4(a)).

3.5 Method validation

The stability indicating assay method was validated for specificity, linearity, precision (inter-day, intra-day and intermediate precision) and accuracy according to ICH guideline Q2 (R1). The specificity of the method was established by determining peak purity for TVT and DPs in a mixture of stressed samples using a photodiode array (PDA) detector and evaluation of the resolution factor. Peak purity was also demonstrated by subjecting all the degradation samples to LC-MS. The PDA and mass detector showed an excellent purity across all peaks, which unambiguously prove the specificity of the method. To establish linearity and range, a stock solution containing 1 mg/mL TVT in mobile phase was diluted to yield solutions in the concentration range of 25-200 µg/ml. The solutions were prepared and analyzed in triplicate. The response for the drug was linear in the investigated concentration range (r^2=0.9992). The linearity data are given in Table S1 (see supplementary information). Table S2 (see supplementary information) shows accuracy data at three different concentrations in triplicate analysis. The recoveries of the added drug were obtained from the difference between peak areas

of fortified and unfortified degraded samples. The recovery of TVT in the presence of degradation products ranged from 99.89 to 100.07%. The intra- and inter-day precisions were determined at three different concentrations 50, 100 and 150 µg/mL, on the same day (*n= 3*) and consecutive days (*n= 3*). Table S2 shows that the %RSD for intra and inter-day precision was < 0.41 % and 0.56 % respectively, indicating that the method was sufficiently precise. Robustness of proposed method was determined by purposely changing the flow rate (0.25 – 0.35 ml/min), column temperature (30 ± 5°C) and change in the % of formic acid in mobile phase (0.1 ± 0.02 %) at three different concentrations (50, 100, 150 µg/ml). Each sample was injected in triplicate (*n=3*), and peak areas obtained were used to calculate means and % RSD values. The %RSD was <1%. No significant changes in assay value were observed by changing these chromatographic conditions which confirms the robustness of the method. The proposed UPLC method was applied for assay of tolvaptan in TOLVAT tablets and results indicated that the amount of tolvaptan was 99.08 ± 0.84 (mean ± S.D) in the tablets corresponds to the requirement of 90–110% of the label claim.

4. Conclusion

The degradation behavior of tolvaptan under various stress conditions was studied. The drug was found to degrade extensively to form 4 degradation products under acid and base hydrolysis and showed only 2 minor degradation products under oxidative degradation. When methanol was used as a co-solvent in stress studies 4 degradation products were formed which were absent in acetonitrile as a co-solvent. This suggests that acetonitrile as a preferred co-solvent for stress degradation studies. All the degradation products were characterized using ESI/MS/MS in combination with accurate mass measurements of product ions and precursors. The degradation pathway of tolvaptan was established.

5. Conflicts of Interest

The authors have no conflicts of interest in this paper.

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Figure Captions:

Figure 1. Overlay of chromatograms for effect of co-solvent [A] Acid hydrolysis, [B] Base hydrolysis, [C] Oxidative degradation

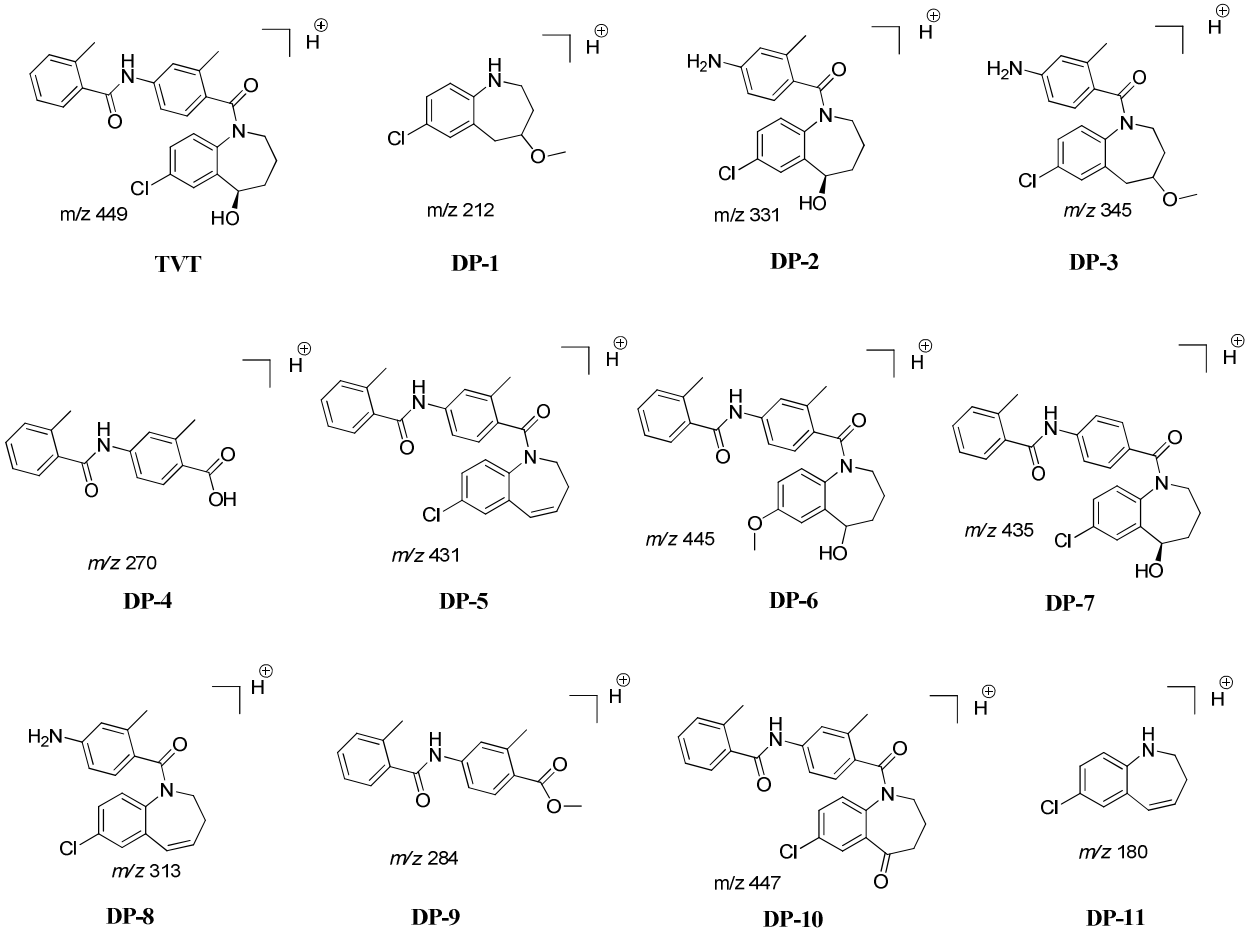
Figure 2. ESI/MS/MS spectrum of (a) **TVT** (*m/z* 449) at 10 eV, (b) **DP-1** (*m/z* 212) at 10 eV, (c) **DP-2 (***m/z* 331) at 10 eV

Figure 3. ESI/MS/MS spectrum of (a) **DP-3** (*m/z* 345) at 15 eV, (b) **DP-4** (*m/z* 270) at 15 eV and (c) **DP-5** (*m/z* 431) at 20 eV

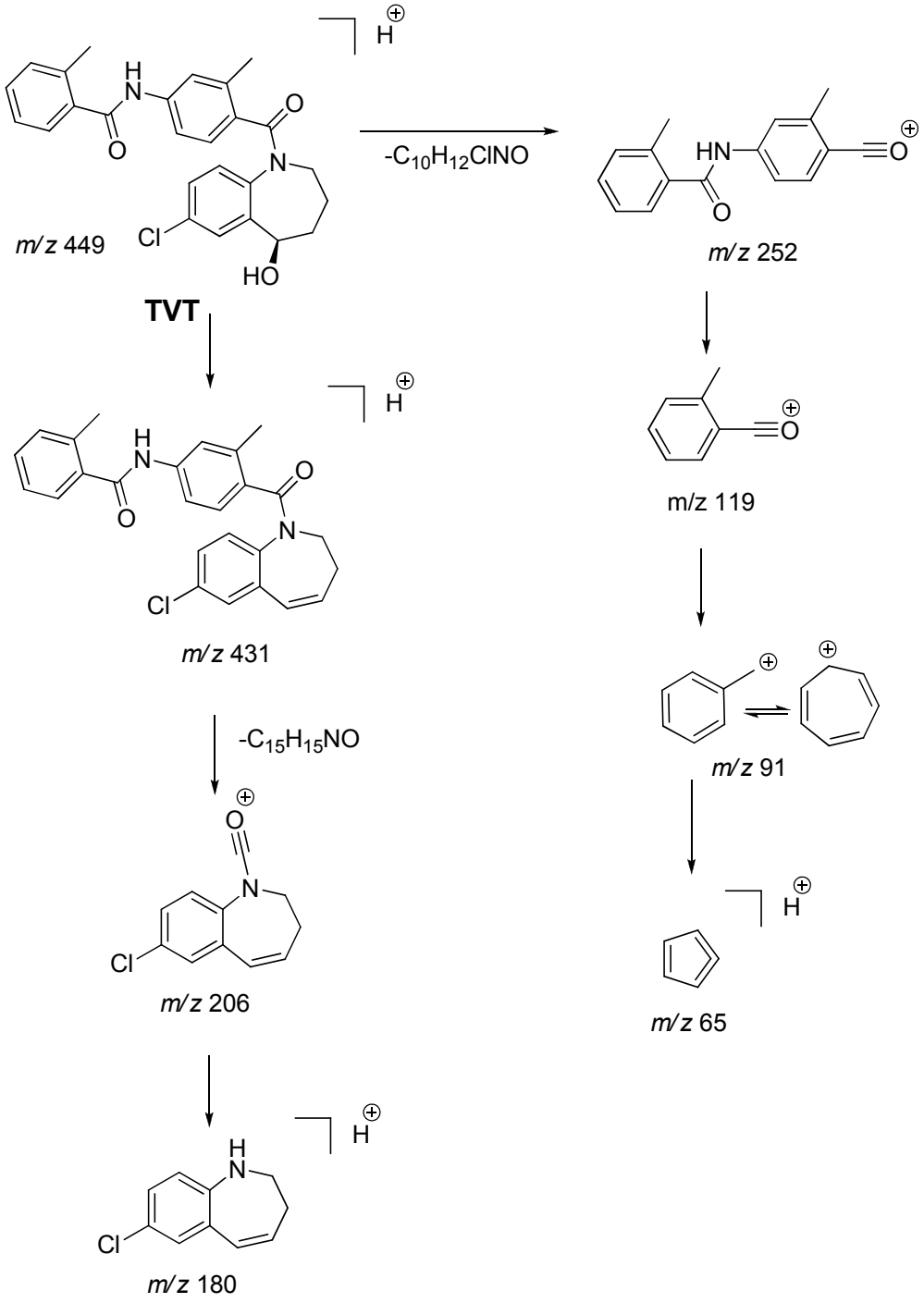
Figure 4. ESI/MS/MS spectrum of (a) **DP-6** (*m/z* 445) at 10 eV, (b) **DP-7** (*m/z* 435) at 15 eV and (c) **DP-8** (*m/z* 313) at 10 eV

Figure 5. ESI/MS/MS spectrum of (a) **DP-9** (*m/z* 284) at 15 eV, (b) **DP-10** (*m/z* 447) at 15 eV and (c) **DP-11** (*m/z* 180) at 15 eV

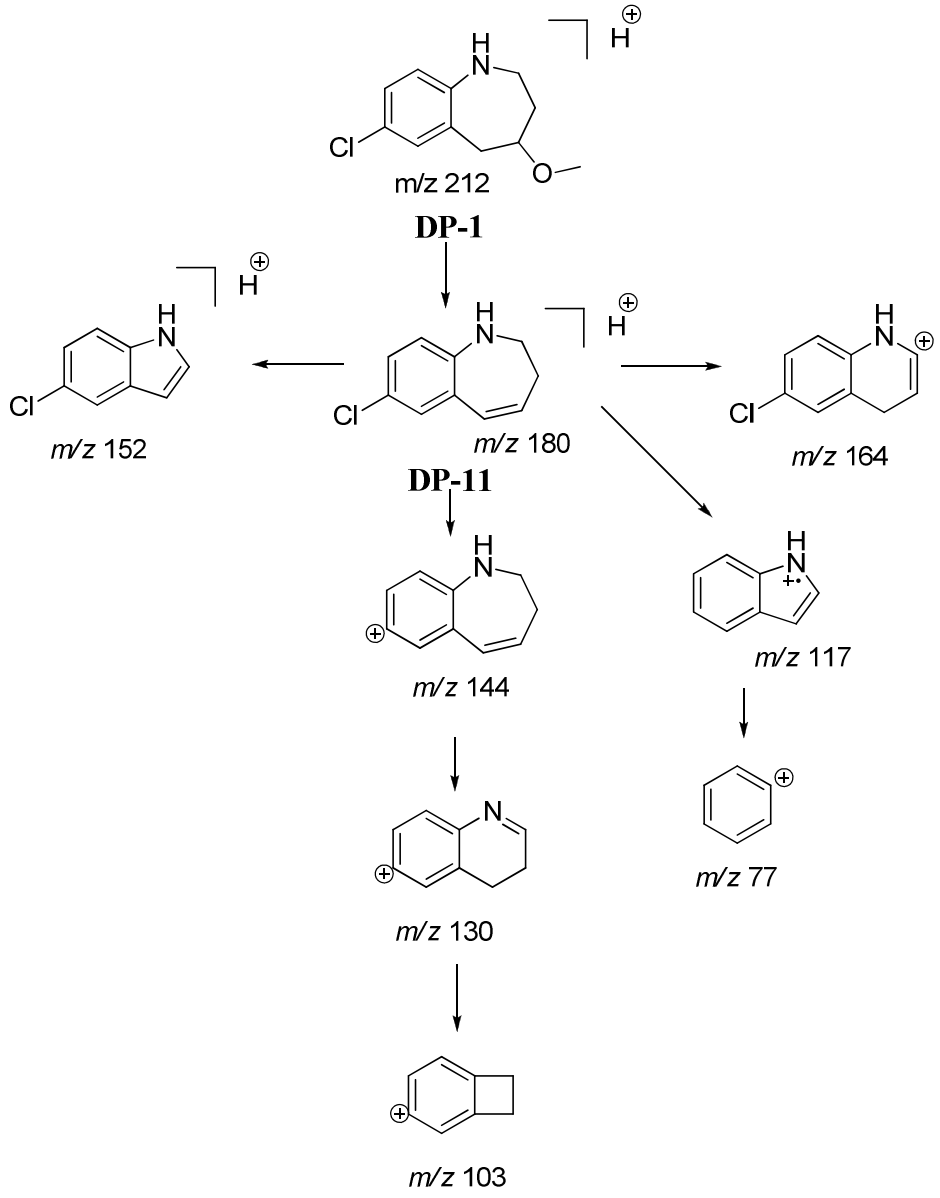
List of tables:



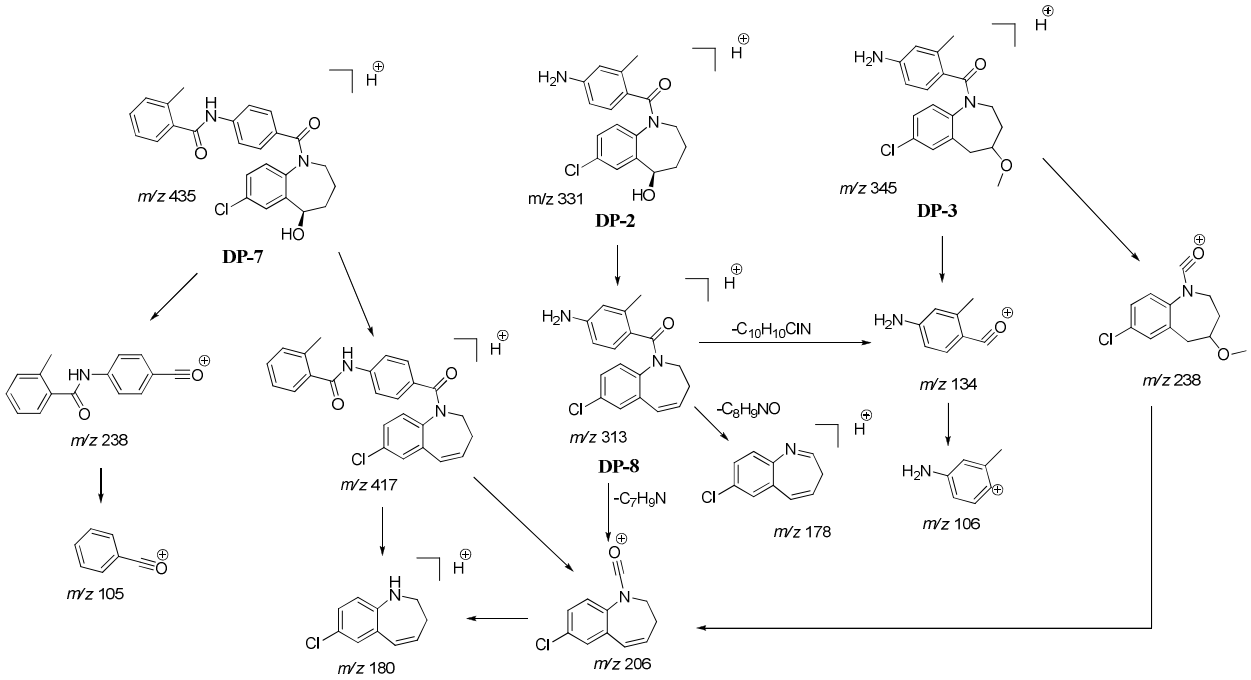
Scheme1. Proposed structures of protonated degradation products of tolvaptan



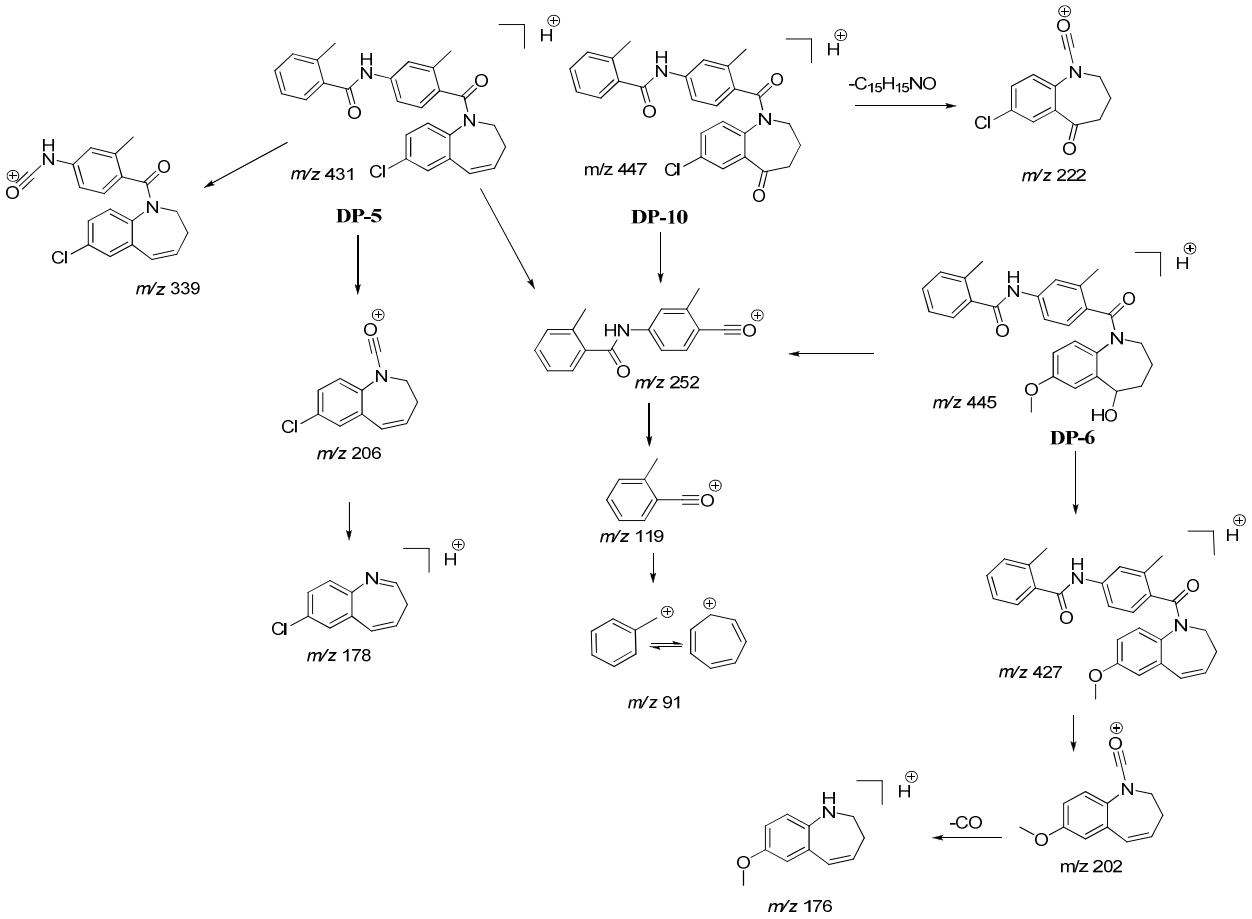
Scheme 2. Proposed fragmentation pathway of protonated tolvaptan



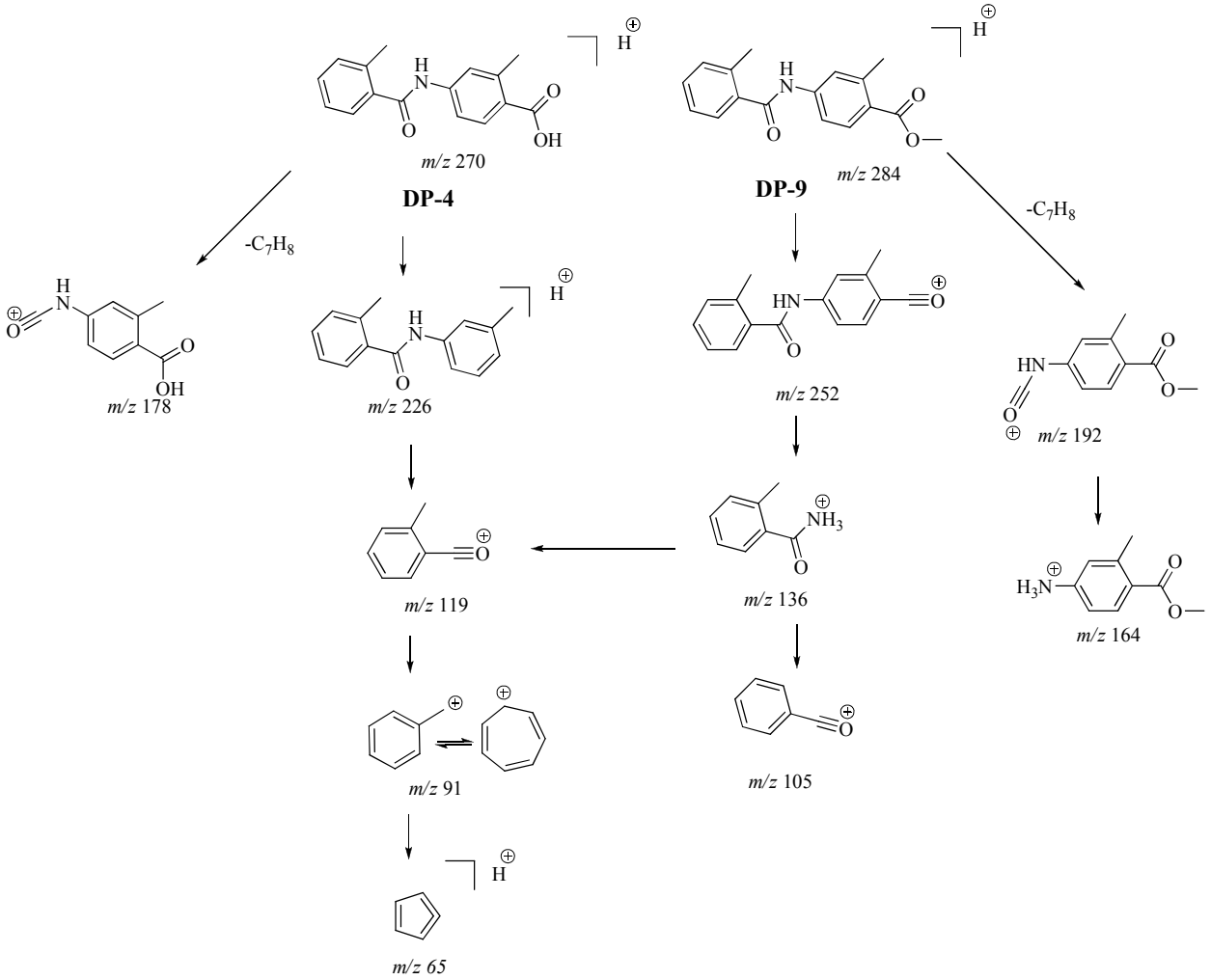
Scheme 3(a). Proposed fragmentation pathway of protonated tolvaptan degradation products (DP-1 and DP-11)



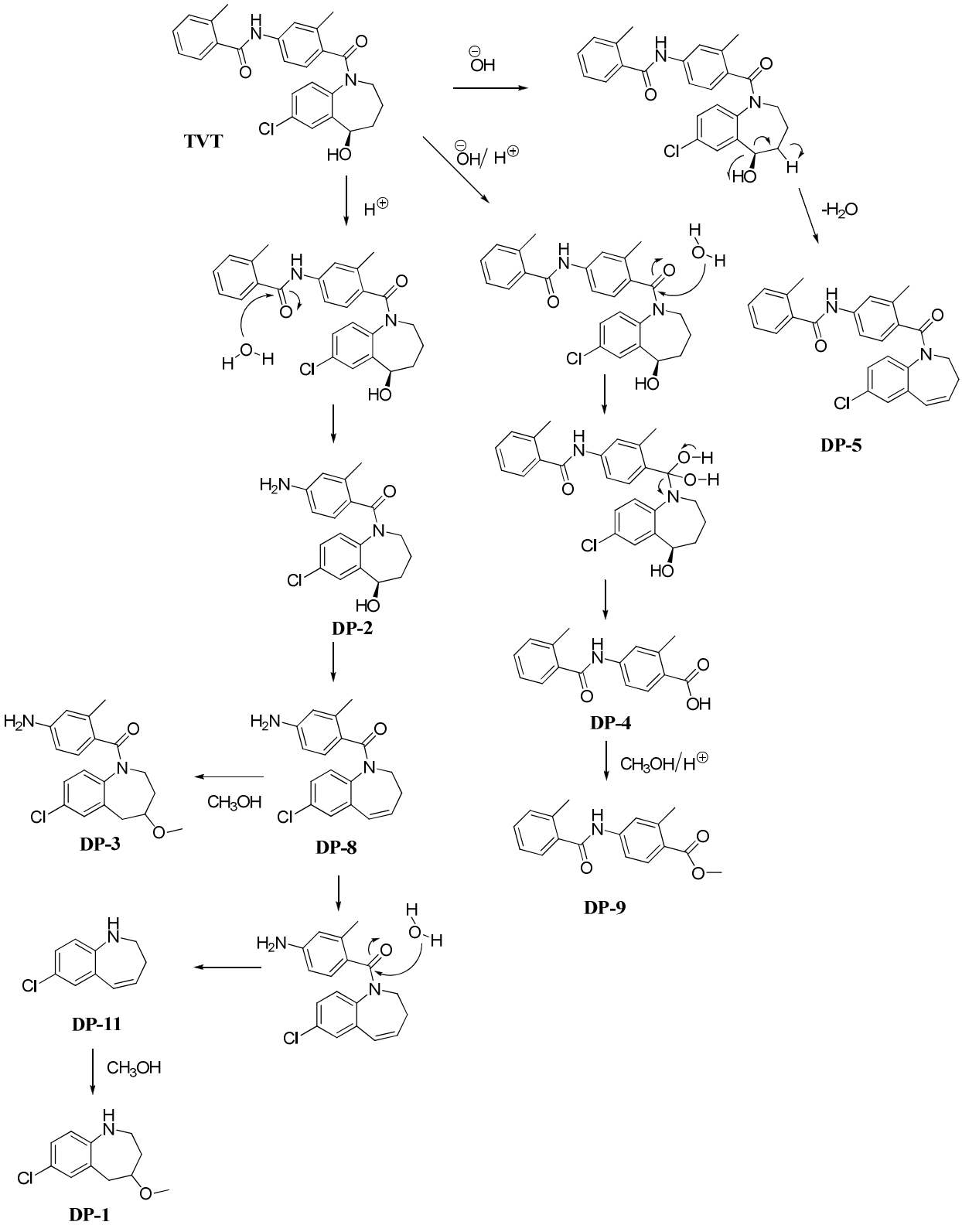
Scheme 3(b). Proposed fragmentation pathway of protonated tolvaptan degradation products (DP-2, DP-3, DP-7 and DP-8)



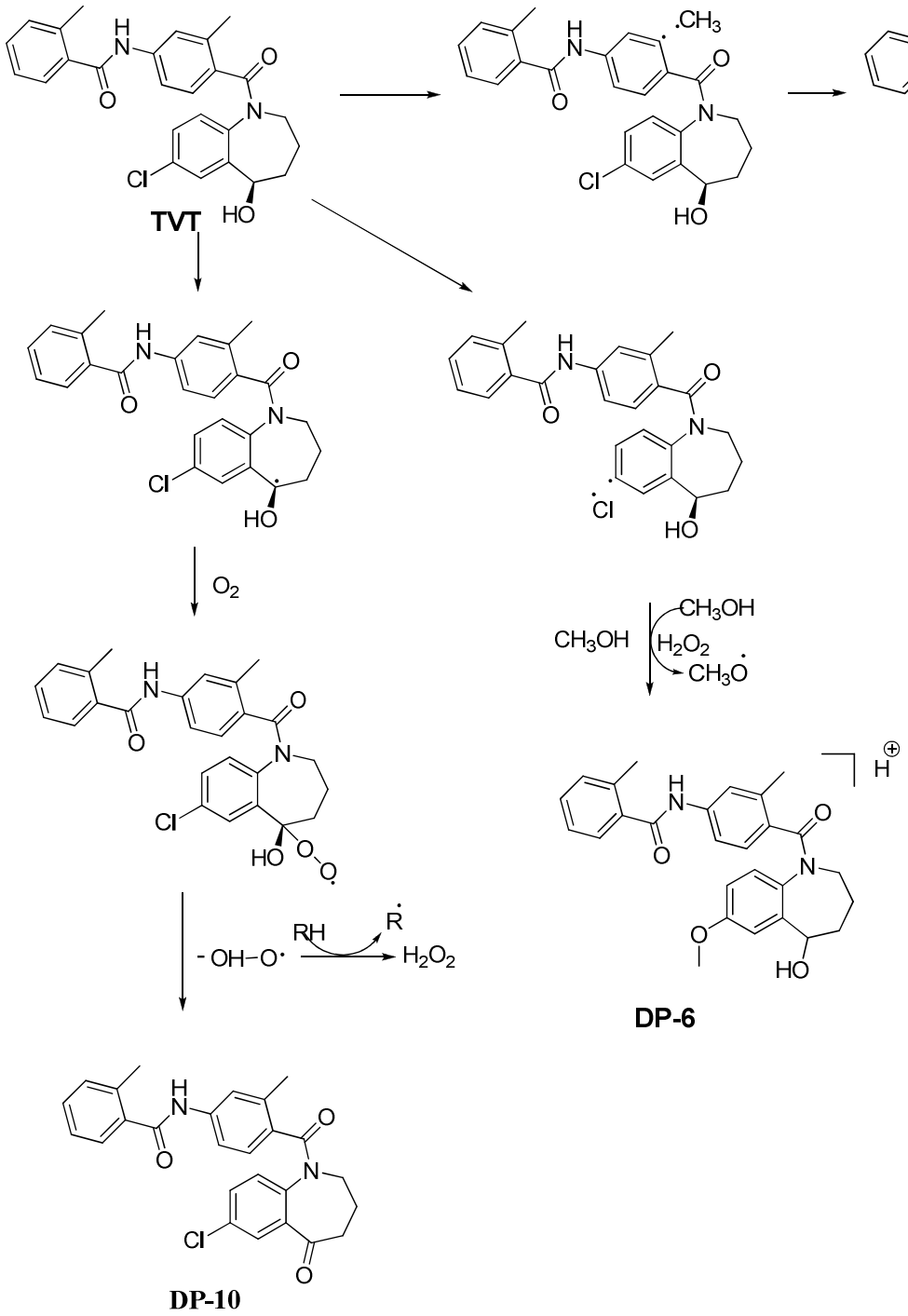
Scheme 3(c). Proposed fragmentation pathway of protonated tolvaptan degradation products (DP-5, DP-6 and DP-10)



Scheme 3(d). Proposed fragmentation pathway of protonated tolvaptan degradation products (DP-4 and DP-9)



Scheme 4(a). Probable mechanisms of formation of DP-1 to DP-5, DP-8, DP-9 and DP-11



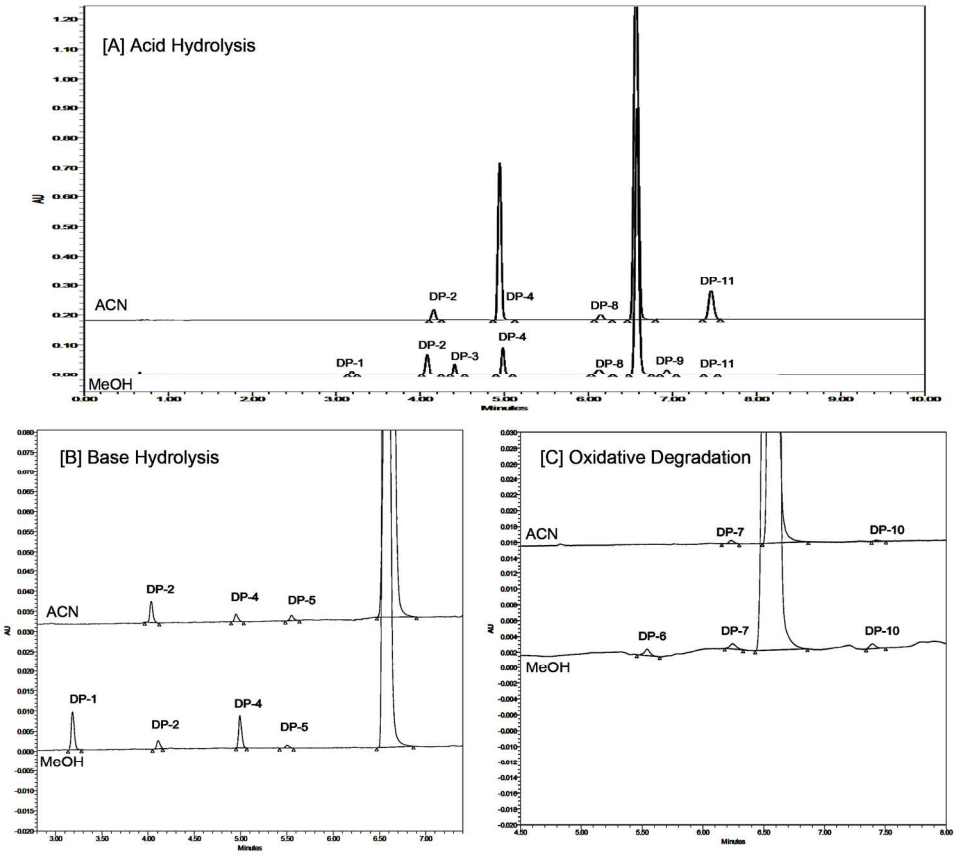
Scheme 4(b). Probable mechanisms of formation of DP-6, DP-7 and DP-10

1 Table 1: MS/TOF data of Tolvaptan and degradation products along with their Elementalcomposition and major fragments

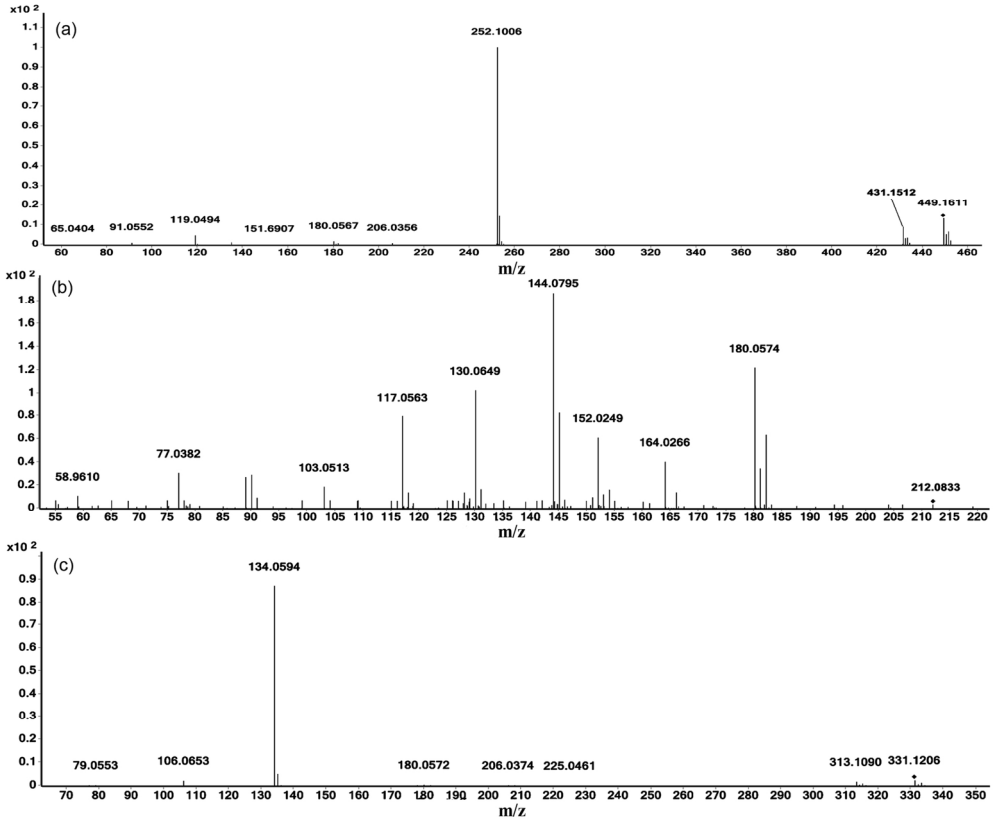
	Retention time (min)	**Molecular Formula**	**Observed m/z**	**Calculated m/z**	**Error**	**ms/ms fragment ions**
TVT	6.55	$C_{26}H_{27}ClN_2O_3^+$	449.1620	449.1626	1.34	431, 252,206, 180, 119, 91, 65
DP-1	3.18	$C_{11}H_{14}ClNO+$	212.0833	212.0837	1.89	180, 164, 152, 144, 130, 117, 103
DP-2	4.08	$C_{18}H_{20}ClN_2O_2+$	331.1206	331.1208	0.60	313, 206, 180, 134
DP-3	4.40	$C_{19}H_{22}ClN_2O_2+$	345.1365	345.1364	-0.29	238, 206, 180, 134, 106
DP-4	4.98	$C_{16}H_{16}NO_3+$	270.1121	270.1125	1.48	226, 178, 119, 91, 65
DP-5	5.48	$C_{26}H_{24}ClN_2O_2+$	431.1535	431.1521	-3.25	339, 252, 206, 178, 119
DP-6	5.53	$C_{27}H_{29}N_2O_4+$	445.2140	445.2122	-4.04	427, 252, 202, 176, 119
DP-7	6.22	$C_{25}H_{24}ClN_2O_3+$	435.1485	435.1470	-3.45	417, 238, 180, 105
DP-8	6.24	$C_{18}H_{17}ClN_2O+$	313.1096	313.1102	1.92	206, 178, 134, 106
DP-9	6.93	$C_{17}H_{18}NO_3+$	284.1273	284.1281	2.82	252, 192, 164, 136, 119, 105
DP-10	7.41	$C_{26}H_{24}ClN_2O_2+$	447.1481	447.1470	-2.46	252, 222, 119, 91, 65
DP-11	7.45	$C_{10}H_{11}ClN+$	180.0585	180.0575	-5.55	164, 152, 144, 130, 117, 103

Table 2. High resolution mass spectrometry (HRMS) data of product ions of protonated tolvaptan and its degradation products

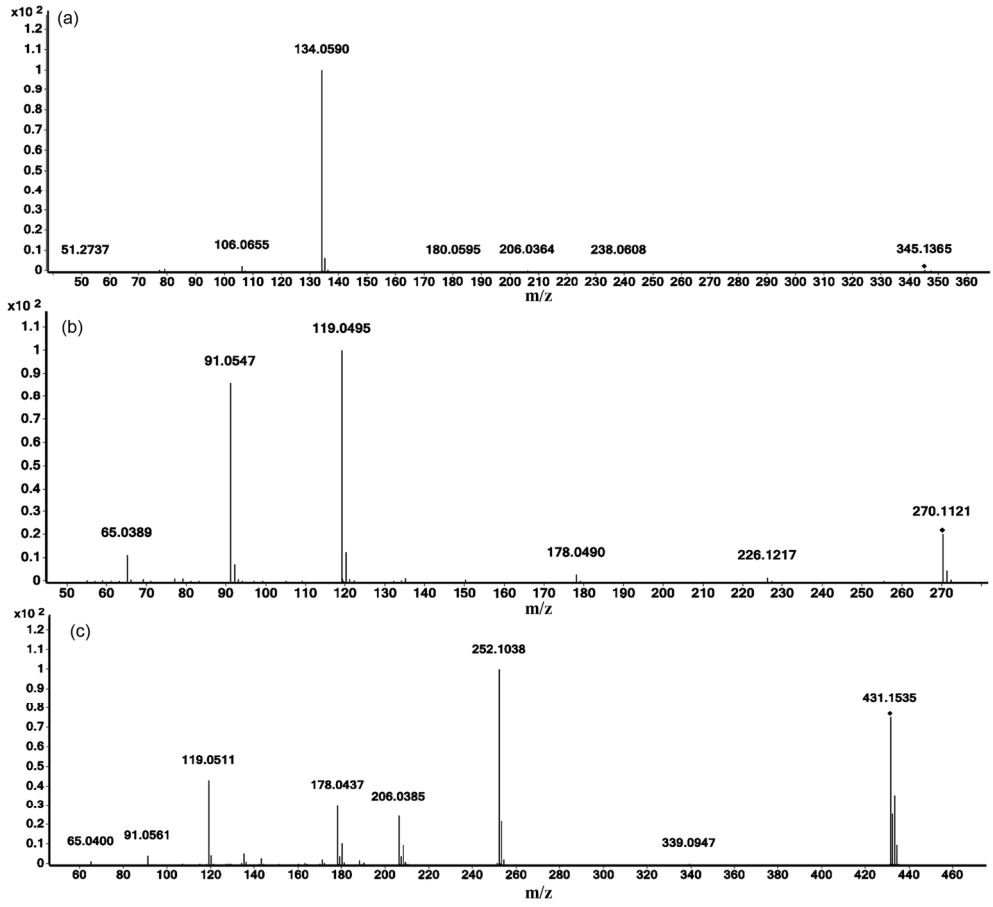
	Molecular Formula	Observed m/z	Calculated m/z	Error
TVT	$C_{26}H_{27}ClN_2O_3^+$	449.1611	449.1626	3.34
	$C_{26}H_{25}ClN_2O_2^+$	431.1512	431.1521	2.09
	$C_{16}H_{14}NO_2$+	252.1006	252.1004	-0.79
	$C_{11}H_9ClNO$+	206.0356	206.0367	5.34
	$C_{10}H_{11}ClN$+	180.0567	180.0575	4.44
	$C_8H_7O^+$	119.0494	119.0491	-2.52
	$C_7H_7^+$	91.0540	91.0542	2.20
DP-1	$C_{11}H_{14}ClNO$+	212.0833	212.0837	1.89
	$C_{10}H_{11}ClN$+	180.0574	180.0575	0.56
	C_9H_7ClN+	164.0266	164.0262	-2.44
	$C_8H_7ClN^+$	152.0262	152.0249	-8.55
	$C_{10}H_{10}N^+$	144.0795	144.0808	9.02
	$C_9H_8N^+$	130.0649	130.0651	1.54
	$C_8H_7N^{+\cdot}$	117.0563	117.0573	8.54
DP-2	$C_{18}H_{20}ClN_2O_2$+	331.1206	331.1208	0.60
	$C_{18}H_{18}ClN_2O$+	313.1090	313.1102	3.83
	$C_{10}H_{11}ClN$+	180.0572	180.0575	1.67
	C_8H_8NO+	134.0594	134.0600	4.48
	C_7H_8N+	106.0653	106.0651	-1.89
DP-3	$C_{19}H_{22}ClN_2O_2$+	345.1365	345.1364	-0.29
	$C_{12}H_{13}ClNO_2^+$	238.0608	238.0629	8.82
DP-4	$C_{16}H_{16}NO_3$+	270.1121	270.1125	1.48
	$C_{15}H_{16}NO^+$	226.1217	226.1226	3.98
	$C_9H_8NO_3$+	178.0490	178.0499	5.05
DP-5	$C_{26}H_{24}ClN_2O_2$+	431.1535	431.1521	-3.25
DP-6	$C_{27}H_{29}N_2O_4$+	445.2140	445.2122	-4.04
	$C_{27}H_{27}N_2O_3$+	427.2036	427.2016	-4.68
	$C_{12}H_{12}NO_2^+$	202.0868	202.0863	-2.47
	$C_{11}H_{14}NO^+$	176.1086	176.107	-9.09
DP-7	$C_{25}H_{24}ClN_2O_3$+	435.1485	435.1470	-3.45
	$C_{25}H_{22}ClN_2O_2$+	417.1360	417.1364	0.96
	$C_{15}H_{12}NO_2$+	238.0887	238.0863	-10.08
	$C_7H_5O^+$	105.0358	105.0335	-21.90
DP-8	$C_{18}H_{17}ClN_2O$+	313.1096	313.1102	1.92
	$C_{10}H_9ClN$+	178.0416	178.0418	1.12
DP-9	$C_{17}H_{18}NO_3$+	284.1273	284.1281	2.82
	$C_{10}H_{10}NO_3$+	192.0643	192.0655	6.25
	$C_8H_{10}NO$+	136.0759	136.0757	-1.47
DP-10	$C_{26}H_{24}ClN_2O_3$+	447.1481	447.1470	-2.46
	$C_{11}H_9ClNO_2$+	222.0311	222.0316	2.25
DP-11	$C_{10}H_{11}ClN$+	180.0585	180.0575	-5.55
	C_9H_7ClN+	164.0269	164.0262	-4.27
	$C_{10}H_{10}N^+$	144.0818	144.0808	-6.94
	$C_9H_8N^+$	130.0649	130.0651	1.54



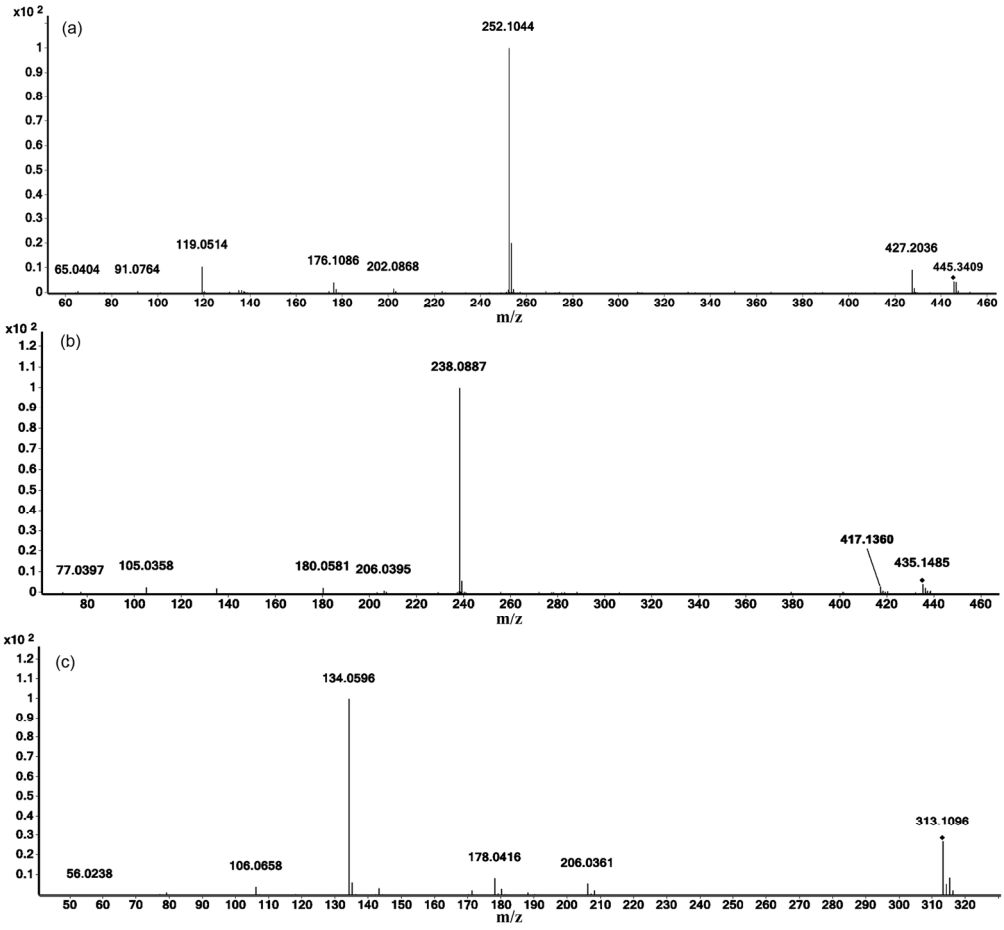
Overlay of chromatograms for effect of co-solvent [A] Acid hydrolysis, [B] Base hydrolysis, [C] Oxidative degradation
199x173mm (300 x 300 DPI)



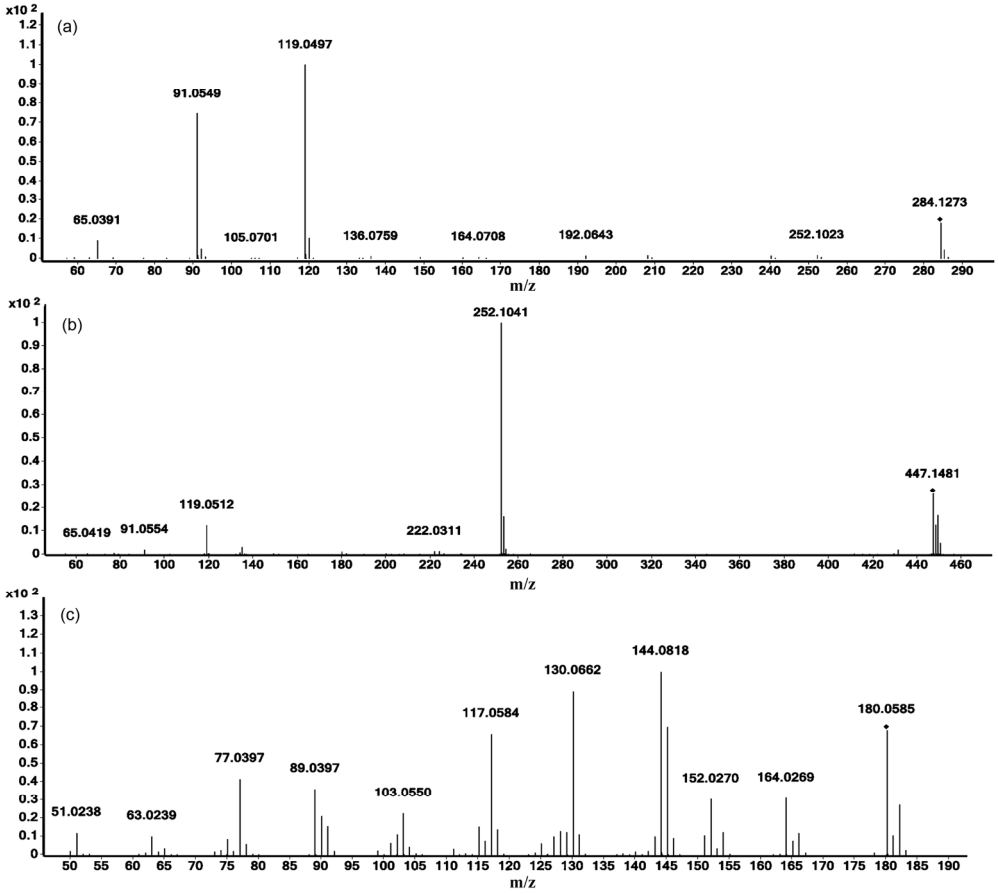
ESI/MS/MS spectrum of (a) TVT (m/z 449) at 10 eV, (b) DP-1 (m/z 212) at 10 eV, (c) DP-2 (m/z 331) at 10 eV
165x136mm (300 x 300 DPI)



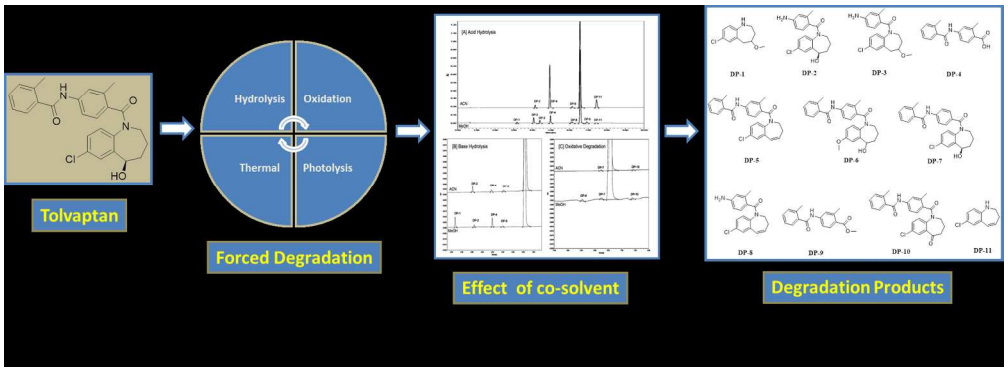
ESI/MS/MS spectrum of (a) DP-3 (m/z 345) at 15 eV, (b) DP-4 (m/z 270) at 15 eV and (c) DP-5 (m/z 431) at 20 eV
184x171mm (300 x 300 DPI)



ESI/MS/MS spectrum of (a) DP-6 (m/z 445) at 10 eV, (b) DP-7 (m/z 435) at 15 eV and (c) DP-8 (m/z 313) at 10 eV
196x182mm (300 x 300 DPI)



ESI/MS/MS spectrum of (a) DP-9 (m/z 284) at 15 eV, (b) DP-10 (m/z 447) at 15 eV and (c) DP-11 (m/z 180) at 15 eV
189x171mm (300 x 300 DPI)



331x119mm (150 x 150 DPI)