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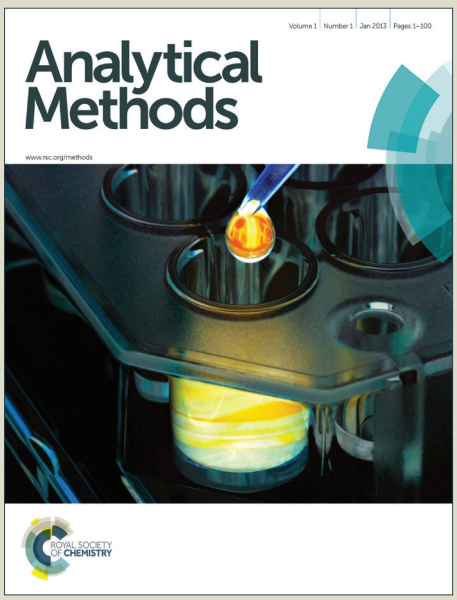

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Study of Degradation Mechanisms of Cyclobenzaprine by LC-MS/MS

Yan Liu[†1], Dantong Zhao[†2], Zhongxi (Zack) Zhao*[1,3]

[1] School of Pharmaceutical Sciences and Center for Pharmaceutical Research & Drug Delivery Systems, Shandong University, Jinan, Shandong 250012, China

[2] Heze Food and Drug Inspection Center of Shandong Province, Heze, Shandong 274000, China

[3] Shandong Academy of Pharmaceutical Sciences, Jinan, Shandong 250101, China

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[†] These authors contributed equally to the work.

* To whom correspondence should be addressed.

Abstract

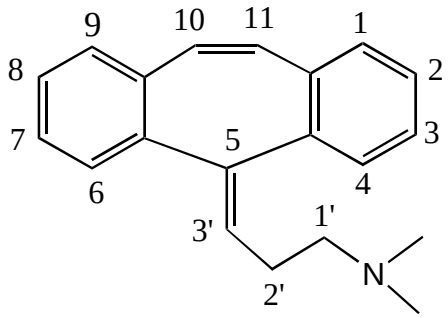
A systematic forced degradation study of cyclobenzaprine (CBA) for the elucidation of the degradation mechanism was carried out in this work to support the new formulation development. Various forced degradation conditions such as acid and base hydrolysis, peroxide oxidation, UV light exposure, high heat and humidity were used to elucidate its degradation profiles. It was then discovered that the protonation of the tertiary amine group of cyclobenzaprine under the acidic condition enabled us to study the oxidation on exocyclic and endocyclic double bonds as well as on the tertiary amine group of cyclobenzaprine in the very short time frame, which overcame the limitation of the common forced degradation study. Liquid chromatography - atmospheric pressure chemical ionization – mass spectrometric technique (LC-APCI-MS) has been used to obtain accurate molecular weight and structural information of cyclobenzaprine and its degradants. A total of fifteen major oxidation products and impurities of cyclobenzaprine were identified and characterized by using LC-MS and LC-MS/MS. These include the bisacid, Cannizzaro degradants, the glycols, the bisaldehyde, ketone derivatives, epoxides, amitriptyline (impurity), the N-oxide, anthraquinone, and dibenzosuberenone. Other techniques such as preparative LC isolation, organic synthesis, photodiode array detector and nuclear magnetic resonance (NMR) spectrometer were also used to obtain the definite structures of the degradants. Our data clearly indicates that cyclobenzaprine degraded through the oxidation of both the endocyclic and exocyclic double bonds to form expoxides as well as oxidation of the tertiary amine group to generate the N-oxide. These unstable expoxides undergo further degradation to more polar compounds and

subsequent cleavage of the alkyl side-chain to form dibenzosuberenone and anthraquinone as the final degradation products.

1. Introduction

Understanding of drug degradation pathways in drug development has been increasingly important due to unprecedented ICH and FDA regulations for the identification of low level degradants and impurities. Screening of potential degradants in drug substances can provide references for the formulation and assay method development to avoid disasters in the late stage of drug development. Liquid chromatography–mass spectrometry (LC-MS) has evolved as a versatile tool for the characterization of drug impurities and degradation products [1-3]. Procedures for generating drug degradants and LC-MS methodologies have been routinely developed in our laboratory to study potential decomposition pathways of drug substances and formulations such as cyclobenzaprine hydrochloride described below.

The original patents of cyclobenzaprine, 3-(5*H*-dibenzo[*a,d*]cycloheptene-5-ylidene)-N,N-dimethyl-1-propanamine, was assigned to Hoffmann-LaRoche & Co. in the late 1950's [4,5]. Later, the hydrochloride salt of cyclobenzaprine was developed as a novel, centrally acting, skeletal muscle relaxant by Merck & Co. [6-10]. Cyclobenzaprine



Cyclobenzaprine

hydrochloride effectively and specifically reduces, or abolishes, excessive tonic muscle activity in several animal models [8,9] as well as in man [10]. There have been a number of reported thin layer (TLC) and gas (GC) chromatographic methods for cyclobenzaprine

HCl [11]. Few HPLC methods for the characterization of cyclobenzaprine HCl drug substance and products have been reported in part due to early drug development having been completed prior to HPLC being a generally accepted chromatographic method and in part due to its poor elution characteristics with old reversed-phase columns [11]. Recently, novel formulations have been developed to meet special patient needs and/or new applications [20-23]. For the renewed interests in novel formulations currently under development, there is a need to study drug degradation by using state-of-art technologies in a systematic manner.

Although there have been several reported papers describing the cyclobenzaprine degradation in vivo (drug metabolism) [12-19], there has been very few published data on the cyclobenzaprine degradation in vitro [11]. Current literature indicates that degradation of cyclobenzaprine occurred primarily by an oxidative process under severe stressed conditions in the aqueous solution. The reported degradants of cyclobenzaprine HCl in acidic aqueous solution identified by HPLC include only exocyclic epoxide, dibenzosuberenone, and anthraquinone [11]. Exocyclic epoxide has been viewed as one of the initial degradants while dibenzosuberenone and anthraquinone have been regarded as the final results of the degradation [11]. A large number of intermediate degradation products have not been identified yet. The elucidation of these intermediate degradants can provide detailed structural evidences for drug degradation pathways.

In this work, a systematic degradation study of cyclobenzaprine under various forced degradation conditions such as acid and base hydrolysis, oxidation, light exposure, high heat and humidity is described. The ideal degradation conditions for cyclobenzaprine were developed and then, low level degradants from the forced degradation studies were

identified and characterized by using on-line LC-UV and LC-MS/MS. Our reversed phase HPLC methods using the new generation of columns provided superior separation of polar degradants, which had not been resolved previously by TLC, GC and HPLC. Trace level degradants have been identified by efficient and sensitive atmospheric pressure chemical ionization (APCI) mass spectrometry. Our results indicate that oxidation was the dominated degradation pathway of cyclobenzaprine HCl. UV spectrometry, isolation, organic synthesis and NMR techniques were also utilized to confirm the structure assignments of the oxidative degradants elucidated from LC-MS/MS results. Finally, the detailed degradation pathways of cyclobenzaprine HCl are proposed from our findings.

2. Experimental

2.1. Chemicals

Cyclobenzaprine hydrochloride（99%）is the product of Suizhou JiaKe Pharmaceutical and Chemical Industry Co., Ltd. (Suizhou, Hubei, China). Dibenzosuberenone (97%) and anthraquinone (98%) were obtained from J&K Scientific Ltd. (Beijing, China). Cyclobenzaprine-10,11-epoxide (95% by area), cyclobenzaprine-10,11-glycols (cis and trans, 99% by the combined area) were synthesized in our laboratory and confirmed by LC-MS and NMR. Amitriptyline hydrochloride (≧98%), trifloroacetic acid (TFA), silver nitrate (ACS reagent, 99+%), ammonia (28% in water), methyl red (ACS reagent) and sodium iodide (99.999%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Methanol and acetonitrile (both HPLC grade) were purchased from Fisher Scientific (Philadelphia, PA). All the standards with the concentrations of 0.1-1 mg mL^{-1} were dissolved in 45% water/55% methanol.

2.2 Forced Degradation Studies

The forced degradation of the acid and base hydrolysis was carried by stressing the cyclobenzaprine drug substance for 6 hours at 60^{o}C in the solutions of 1 N HCl and NaOH, respectively. The peroxide oxidation testing was conducted by stressing the cyclobenzaprine drug substance for 12 hours at room temperature in 3% H_2O_2. For stress testing in the solid state, The solid-state photostability study of the cyclobenzaprine drug substance was completed by following the UV-visible light conditions stated in the option 2 of the ICH photostability guidelines [24]. The forced degradation of cyclobenzaprine under the high heat and humidity included the stressing the drug powder for up to 6 months under a high temperature of 40^{o}C and relative humidity (RH) of 75%.

A compound specific oxidation procedure under the acidic condition was as following: the sample solution was prepared by dissolving about 2 mg mL^{-1} of cyclobenzaprine HCl in 0.1% TFA along with 1% hydrogen peroxide as an oxidant. A short piece of a stainless steel wire was put in the reaction mixture as a catalyst. The ideal oxidation product mixture with a reasonable amount of degradants for identification could be generated within a couple of hours with the metal wire. Otherwise, producing similar solution mixture needs at least four days.

2.3 Analytical Liquid Chromatography

An Agilent liquid chromatographic instrument equipped with a diode array detector (Model Infinity 1260, Agilent Technologies, CA, USA) was used. The HPLC Column thermostated at 25℃ was Phenomenex Kinetex XB-C18 (100 x 4.6 mm, 2.6 μm, from

Phenomenex, CA, USA). Mobile phases consisted of 0.1% formic acid (A) and acetonitrile (B) with a flow rate of 1.0 mL/min. A linear gradient scheme was as following: 0 - 8 minutes from 20%-30% B, 8-18 minutes from 30%-40% B, 18-25 minutes from 40-50% B, 25-26 minutes from 50-20% B, and finally equilibration time of 10 minutes at 20%B. Injection volume was 10 μL and UV detection wavelength was set at 254 nm.

2.4 Liquid Chromatography-Mass Spectrometry

The mass spectrometer utilized in all studies was an API 5000™ (Applied Biosystems, Foster City, CA, USA) triple quadruple instrument equipped with both atmospheric pressure chemical ionization (APCI) and electrospray ionization sources. A positive ion mode was utilized in these experiments. The HPLC separation was done on a Shimadzu Model LC-20ADXR solvent delivery system equipped with a Model SIL-20AXR autosampler (Shimadzu Corp., Kyoto, Japan). The optimal APCI-MS/MS parameters were as follows: Source temperature was 450°C; the flow rates for curtain gas and collision gas were set at 15 and 6 psi, respectively. The nebulizer current was at 2.0 μA. Declustering potential, collision energy, entrance potential and collision cell exit potential were set at 100 V, 23 eV, 10 V and 13 V, respectively, for cyclobenzaprine and its degradants. Dwell Time was set at 100 ms. The HPLC Column thermostated at 25℃ was Phenomenex Kinetex XB-C18 (100 x 4.6 mm, 2.6 μm, from Phenomenex, CA, USA). Mobile phases consisted of 0.1% formic acid (A) and acetonitrile (B) with a flow rate of 1.0 mL/min. A linear gradient scheme was as following: 0-8 minutes from 20%-30% B, 8-18 minutes from 30%-40% B, 18-25 minutes from 40-50% B, 25-26 minutes from 50-20% B, and finally equilibration time of 10 minutes at 20%B. Injection volume was 10 μL.

2.5 Preparative Liquid Chromatography

A Waters Model 600 LC pump equipped with a Model 996 photodiode array detector was used. HPLC Column was Waters μBondapak C18 with the dimension of 300 x 21 mm (10 μm particle size). Mobile phases consisted of (A) 0.1% formic acid in 25% acetonitrile/75% water and (B) 0.1% formic acid in acetonitrile with a flow rate of 20 mL/min^{-1}. Gradient conditions were as following: 15 min at 5%B, 15 min from 5%B to 28%B, 2 min from 28%B to 60%B and finally 8 min at 60%B. Column equilibration time was 8 min. The chromatography was optimized for the best separation of early eluting species. Injection volume was 2 mL and UV detection wavelength was 254 nm.

2.6 Synthesis of Cyclobenzaprine 10,11-Epoxide and its Glycols

A solution of cyclobenzaprine in methylene chloride was stirred and cooled in an ice bath as the solution of peracetic acid in methylene chloride was added drop wise. The resulting mixture was stirred in the ice bath for additional 20 min. The mixture was washed with 10% $NaHCO_3$ three times and saturated NaCl solution. The organic layer was dried (Na_2SO_4) and the solvent was removed on a rotary evaporator to give cyclobenzaprine 10,11-epoxide [25]. The conversion from cyclobenzaprine 10,11-epoxide to cyclobenzaprine 10,11-glycols was completed by the acidic hydrolysis under the dilute HCl. The identities and purities of the products produced were conveniently determined by ^1H NMR and LC-MS.

3. Results and Discussion

A systematic degradation study of cyclobenzaprine under various forced degradation conditions was carried out on the basis of our laboratory tradition. The acid and base hydrolysis, and peroxide oxidation of cyclobenzaprine were conducted for the stress testing in the solution state while the stress testing of cyclobenzaprine under the UV-visible light exposure, high heat and humidity was performed in the solid state. Major degradants identified during various preliminary forced degradation studies are summarized in Table 1.

The results from these preliminary forced degradation studies described above suggest that the oxidation on the tertiary amine group was the major degradation pathway for cyclobenzaprine. However, the number of potential degradants (3 degradants) of cyclobenzaprine observed from this initial forced degradation study was far smaller than those (12 degradants) found in the aged innovator's product (data not shown), indicating that the initial forced degradation conditions adopted from the common strategies were not sufficient. It was then discovered that the protonation of the tertiary amine group of cyclobenzaprine under an acidic condition enabled us to study the oxidation on exocyclic and endocyclic double bonds as well as on the tertiary amine group of cyclobenzaprine in the very short time frame. Liquid chromatography - atmospheric pressure chemical ionization– mass spectrometric technique (LC-APCI-MS) has been used to obtain accurate molecular weight and structural information of cyclobenzaprine and its degradants. A total of fifteen major oxidation products and impurities of cyclobenzaprine were identified and characterized by using LC-MS and LC-MS/MS.

The total ion chromatogram of cyclobenzaprine HCl in the acidic aqueous solution using LC-APCI-MS is shown in Figure 1. Degradants **XIII** and **XIV** have extremely weak MS responses, which are labeled in dot lines. A total of fifteen oxidation products and impurities were detected and their major tandem MS fragments and UV absorption maxima are listed in Table 2. The detailed structural determination of these degradants and impurities identified is described below.

3.1 Fragmentation Analysis of Cyclobenzaprine and Amitriptyline

The tandem mass spectrum of cyclobenzprine (CBA) is briefly analyzed in order to help the discussion of degradant identification. As is shown in Figure 2a, the major fragments of CBA are at *m/z* 231 [$MH^+ - HN(NH_3)_2$], 216 [$MH^+ - C_6H_4$], 205 [231 – C_2H_2], 191, 84 [$+CHCHCH_2N(CH_3)_2$] and 58 [$+CH_2N(CH_3)_2$]. Three UV adsorption maxima of CBA were observed at 225, 245 (shoulder) and 290 nm. The CBA UV adsorption at 245 nm and 290 nm is attributed to the adsorption of exocyclic and endocyclic double bonds, respectively. The identification of the process impurity amitriptyline (AMT) is also discussed here due to its structural similarity to CBA (Figure 2b). Amitriptyline has a molecular ion at *m/z* 278, which is 2 amu higher than that of CBA. Major fragments of amitriptyline were observed at *m/z* 233 [$MH^+ - HN(NH_3)_2$], 218 216 [$MH^+ - C_6H_4$], 205, 191, 179, 155, 117, 105, 91, 84 and 58. Its identity was quickly confirmed by using the authentic standard of amitriptyline.

3.2 Degradant I - Bisacid

The LC-MS/MS mass spectrum and proposed fragmentation pattern of Degradant **I** are shown in Figure 3. The mass spectrum shows the protonated molecular ion [MH^+] at *m/z* 340 which are 64 amu higher than CBA (*m/z* 276). The molecular weight is corresponding with the introduction of two carboxylic acid (COOH) groups. The characteristic fragment ions were observed at m/*z* 322 [MH^+ - H_2O], 304 [322 - H_2O], 277 [322 - $HN(CH_3)_2$], 259 [277 - H_2O], 231 [259 - CO], 221 [259 – C_3H_2], 193 [221 - CO], 179 [205 – CHCH], and 58 [$+CH_2N(CH_3)_2$]. The fragments at *m/z* 58 indicate that the tertiary amine side chain is intact and the modification is on the other end of CBA. The UV spectrum of Degradant **I** shows only a broad maximum at 240 nm. The absence of 290 nm absorption (from endocyclic double bond) suggests that the 10,11-double bond is absent in Degradants **I**. The UV and MS/MS results suggest that Degradant **I** is a bisacid degradant, which may be formed from the further oxidation of the diol derivatives (Degradants **II** and **III**).

3.3 Degradants II, III and VII - Glycol Derivatives

The tandem mass spectrum and proposed fragmentation pattern of Degradant **II** are shown in Figure 4a. The mass spectrum shows the protonated molecular ion [MH^+] at *m/z* 310, which are 34 amu higher than CBA (*m/z* 276). The molecular weight is corresponding with the introduction of two hydroxyl (OH) groups. Characteristic fragment ions were observed at m/*z* 292 [MH^+ - H_2O], 264 [292 - CO], 247 [MH^+ - $HN(CH_3)_2$], 229 [247 - H_2O], 219 [247 - CO], 203 [229 -CHCH], 191 [219 - CH_2CH_2], 84 [$+CHCHCH_2N(CH_3)_2$], and 58 [$+CH_2N(CH_3)_2$]. The fragments at *m/z* 58 and 84 indicate that the tertiary amine side chain is intact and the modification is on the other end of CBA. The fragments at *m/z* 292 and

229 generated from the dehydration of the related ions indicate that Degradant **II** is not a phenolic compound. The ions at *m/*z 264 and 219 were produced by losing carbon monoxide from the pre-cursor ions, indicating that the hydroxyl group(s) is added on C-10 and/or C-11 position(s). The UV spectrum of Degradant **II** showed only a broad maximum at 245 nm. The absence of 290 nm absorption (from C-10 and C-11 conjugation) suggested that the 10,11-double bond is absent in Degradant **II**. The UV properties of the product are consistent with the MS/MS results. Hence, the structure of Degradant **II** is cyclobenzaprine-10,11-glycol. Degradant **III** (Peak #2 in Figure 1) has the same molecular weight and MS and MS/MS spectra as Degradant **II**, indicating that Degradant **III** is an isomer of Degradant **II**. By injecting the authentic standards under the same chromatographic conditions, it was found that Degradants **II** and **III** are cis- and trans-cyclobenzaprine-10, 11-glycol, respectively, which are presumably formed from the hydrolysis of the epoxide degradant (Degradant **IX**).

Degradant **VII** also has the molecular weight of 309 and its tandem mass spectrum and proposed fragmentation are shown in Figure 4b. The major fragments of Degradant **VII** are observed at *m/z* 292 [MH^+ - H_2O], 274 [292 - H_2O], 247 [MH^+ - $HN(CH_3)_2$], 229 [247 - H_2O], 203 [229 -CHCH], 193, 72 [$+CH_2CH_2N(CH_3)_2$], and 58 [$+CH_2N(CH_3)_2$]. Fragment ions at *m/z* 72 and 84 are one of the different fragment pairs in the tandem mass spectra of Degradants **VII** and **II**. Fragment at *m/z* 72 may suggest that the exocyclic double bond in CBA be modified in Degradant **VII**. The UV spectrum of Degradant **VII** clearly supports the MS structural assignment because it has the 290-nm adsorption (endocyclic double bond) and lack of the 245-nm maximum (exocyclic double bond). So, Degradant **VII** was

assigned as cyclobenzaprine-3',5-diol, which may be formed through the hydrolysis of the corresponding epoxide (Degradant **XI**).

3.4 Degradants IV and V - Cannizzaro Degradants

The tandem mass spectrum of Degradant **IV** (Peak #3 in Figure 1) is shown in Figure 5. The molecular weight [MH^+ - H^+] of Degradant **IV** was determined to be 325 by LC-ESI-MS. Characteristic fragment ions are present at *m/z* 308 [MH^+ - H_2O], 280 [308 - CO], 245, 229, 219, 203 [229 – CHCH], 191 [217 - CHCH], 84 [$+CH_2CH_2N(CH_3)_2$], and 58 [$+CH_2N(CH_3)_2$]. The presence of the amine side chain ions at *m/z* 58 and 84 suggests that the side chain is still intact on the Degradant **IV**. The UV spectrum of Degradant **IV** shows the maxima at about 240 nm, indicating that the endocyclic double bond (290 nm absorption) is absence. So, the proposed structure of Degradant **IV** is the Cannizzaro degradant (Figure 5), which may be formed through the Cannizzaro oxidation of the bisaldehyde degradant (Degradant VI below). Degradant **V** (Peak #4 in Figure 1) has the same molecular weight (325 amu) as Degradant **IV**. Majority tandem fragment ions are also identical between these two degradants, indicating that Degradants **IV** and **V** are the positional isomers.

3.5 Degradants VI - Bisaldehyde

The tandem mass spectrum of Degradant **VI** (Peak #5 in Figure 1) is shown in Figure 6. The molecular weight of Degradant **VI** was determined to be 307, corresponding to the introduction of two oxygen atoms to CBA (MW 275). Characteristic fragment ions (see Figure 5) are present at *m/z* 290 [MH^+ - H_2O], 280 [MH^+ - CO], 245 [290 - $HN(CH_3)_2$], 229,

217 [245 - CO], 191 [217 - CHCH], 72 [+$CH_2CH_2N(CH_3)_2$], and 58 [+$CH_2N(CH_3)_2$]. The presence of the amine side chain ions at *m/z* 58 and 72 suggest that the side chain remains unchanged on Degradant **VI**. The UV spectrum of Degradant **VI** shows the broad maxima at 235 and 260 nm, indicating that the endocyclic double bond is absence. The possible structure of Degradant **VI** was determined to be the bisaldehyde, which has been further confirmed by the positive Trollen (Aldehyde) test.

3.6 Degradants VIII and X - Monoketo Derivatives

The mass spectrum of Degradant **VIII** exhibited a molecular ion [MH^+] at *m/z* 292, indicating addition of an oxygen atom to CBA. Characteristic fragment ions (Figure 7) were observed at m*/z* 247 [MH^+ - $HN(CH_3)_2$], 229 [247 - H_2O], 219 [247 - CO], 203 [229 -CHCH], 191 [219 - CH_2CH_2], 179, 91, 84 [+$CHCHCH_2N(CH_3)_2$], and 58 [+$CH_2N(CH_3)_2$]. The tertiary amine side chain remains unchanged as indicated by the presence of the fragment ions at *m/z* 58 and 84. Degradant **VIII** is not a phenolic derivative as is suggested by both fragments at *m/*z 229 formed by dehydration and at *m/*z 219 generated by eliminating carbon monoxide. This indicates that oxygen atom should be attached to either exocyclic or endocyclic double bonds. Two UV adsorption maxima at about 224 and 255 nm suggest the absence of endocyclic double bond (290-nm absorption). So, Degradant **VIII** was determined to be 10 or 11-keto-cyclobenzaprine. In the similar fashion, Degradant **X** is assigned as the isomer of Degradant **VIII** because both of them have the identical MS/MS and UV spectra.

3.6. Degradants IX and XI - Epoxides

The mass spectrum of Degradant **IX** (Peak #8 in Figure 1) corresponds to a compound with the molecular ion [MH^+] at *m/z* 292, identical to that of the monoketo derivatives. However, there are some different features (see Figures 7 and 8a) in the fragmentation pathways between Degradant **IX** and the monoketo derivatives. Characteristic fragment ions of Degradant **IX** (Figure 8a) were observed at m/*z* 264 [MH^+ - CO], 247 [MH^+ - $HN(CH_3)_2$], 233 [MH^+ - $N(CH_3)_3$], 219 [247 - CO], 207 [233 -CHCH], 191 [219 - CH_2CH_2], 178, 84 [+CHCHCH2$N(CH_3)_2$], and 58 [+$CH_2N(CH_3)_2$]. The amine side chain is intact as is suggested by the presence of the ions at *m/z* 58 and 84. Only the ions at *m/*z 264 and 219 generated by eliminating carbon monoxide were observed, suggesting that Degradant **IX** be an epoxide derivative. The absence of the 290-nm absorption (derived from C-10 and C-11 conjugation) suggests that the endocyclic double bond is modified in Degradant **IX**. So, Degradant **IX** was identified to be cyclobenzaprine-10,11-epoxide (endo-epoxide), which has been also confirmed by the authentic standard using both MS and HPLC techniques.

Degradant **XI** is another degradant with the addition of oxygen atom (Figure 8b). The intense fragment ions of Degradant **XI** were observed at m/*z* 247 [MH^+ - $HN(CH_3)_2$], 229 [247 - H_2O], 221 [247 - CHCH], 203 [229 - CHCH], 193 [221 - CO], 72 [+$CH_2CH_2N(CH_3)_2$], and 58 [+$CH_2N(CH_3)_2$]. Here, the amine side chain remains unchanged as is indicated by the presence of the ions at *m/z* 58 and 72. The unique fragments at *m/z* 221 and 193 suggest that the exocyclic double bond be oxidized. The presence of the 290-nm absorption (derived from C-10 and C-11 conjugation) in the UV spectrum indicates that the endocyclic double bond is intact. Finally, the positive epoxide test further confirms that Degradant **XI** is cyclobenzaprine-3',5-epoxide (exo-epoxide).

3.7 Degradant XII – N-oxide

The mass spectrum of Degradant **XII** corresponds to a compound with the molecular ion [MH^+] at *m/z* 292 and mass increase of 16 from the parent. Characteristic fragment ions of Degradant **XII** (Figure 9) were observed at m/*z* 274 [MH^+ - H_2O], 262 [MH^+ - HCHO], 231 [MH^+ - $HON(CH_3)_2$], 216 [MH^+ – C_6H_4], 191 [231 – C_3H_4], 178, 100 [$CHCHCH_2(CH_3)_2NO$], and 74 [$CH_2(CH_3)_2NO$]. The UV absorption maxima at 225, 245 and 290 nm for Degrarant **XII** observed were the same those for cyclobenzaprine, indicating that both endocyclic and exocyclic double bonds are intact and the modification site was most likely in the side chain – the tertiary amine oxidation. In addition, the identity of Degrarant **XII** was also confirmed to be cyclobenzaprine N-oxide by a purified standard isolated by the preparative HPLC from the peroxidation of cyclobenzaprine under the neutral pH condition.

3.8. Degradants XIII and XIV - Side-Chain Decomposition Degradants

Both Degradants **XIII** and **XIV** have very week MS responses under APCI and/or electrospray ionization conditions and so, their peaks are labeled in the dot lines in Figure 1. Degradants **XIII** and **XIV** were identified as anthraquinone and dibenzosuberenone, respectively, by using MS and LC-UV methods with the corresponding authentic standards. Degradant **XIV** may be formed from the side-chain decomposition of CBA and related degradants. Degradant **XIII** could be formed from the further oxidization of dibenzosuberenone (Degradant **XIV**).

3.8. Proposed degradation mechanism

Figure 10 shows the possible degradation pathways of cyclobenzaprine. The results suggest that cyclobenzaprine may degrade through three major oxidation pathways. Firstly , the oxidation of the endocyclic double bond produces endo-epoxide (Degradant **IX**), which may be hydrolyzed into cyclobenzaprine-10,11-glycols (Degradants **III** and **IV**). These glycols is then transformed into dialdehyde (Degredate **VI**), which can be oxidized into diacid (Degradant **I**) through the Cannizzaro degradants (**IV** and **V**). Secondly, the exocyclic double bond is oxidized into the exo-epoxide (Degradant **XI**); the exo-epoxide is hydrolyzed into the 3',5-glycol (Degradant **VII**) that may be oxidized into dibenzosuberenone (Degradant **XIV**) and anthraquinone (Degradant **XIII**) as the final products. Thirdly, the oxidation of the tertiary amine side chain is to form cyclobenzaprine N-oxide (Degradant **XII**). Thus, the final result would be a large number of different oxidation products presented at trace concentrations.

Conclusions

In this work, a systematic degradation study of cyclobenzaprine (CBA) was carried out. A total of fifteen major oxidation products and impurities of CBA were identified and characterized by using LC-MS and LC-MS/MS and other techniques. These degradants are believed to be formed from the primary oxidation of the endo- and exo-cyclic double bonds as well as the tertiary amine side chain on CBA and from the subsequent degradation of the primary oxidative degradants. The main difference between the *in vitro* degradation and *in vivo* metabolism pathways of CBA is related to the absence of the aromatic ring oxidation in the *in vitro* degradation. Our experimental data basically confirm a major portion of the hypothesis of CBA degradation proposed by Cotton and Down [11].

The confirmed degradation mechanisms of cyclobenzaprine will provide a basis for further pharmaceutical analytical and formulation development.

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References

1. S. Singh, T. Handa, M. Narayanam, A. Sahu, M. Junwal, R.P. Shah, A critical review on the use of modern sophisticated hyphenated tools in the characterization of impurities and degradation products, J. Pharm. Biomed. Anal. 69 (2012) 148–173.
2. B. Pramanik, M.S. Lee, and G. Chen (Eds.), Characterization of Impurities and Degradants Using Mass Spectrometry, Published by John Wiley & Sons, Inc., Hoboken, New Jersey, USA, 2011.
3. D.Q. Liu, M. Sun, A.S. Kord, Recent advances in trace analysis of pharmaceutical genotoxic impurities, J. Pharm. Biomed. Anal. 51 (2010) 999–1014.
4. F. Hoffmann-LaRoche & Co., Swiss patent 356,759.
5. F. Hoffmann-LaRoche & Co., British patent 858,187(1961).
6. N.N. Share, French patent 2,100,873(1972).
7. C.D. Barnes and W.L. Adams, Effects of cyclobenzaprine on interneurones of the spinal cord, Neuropharmcology 17 (1978) 445-450.
8. J.V. Basmajian, Cyclobenzaprine hydrochloride effect on skeletal muscle spasm in the lumbar region and neck: two double-blind controlled clinical and laboratory studies, Arch. Phys. Med. Rehabil. 59 (1978) 58-63.
9. B.R. Brown and J. Womble, Cyclobenzaprine in Intractable Pain Syndromes With Muscle Spasm, J.A.M.A. 240 (1978) 1151-1152.
10. N.N. Share and C.S. McFarlan, Cyclobenzaprine: novel centrally acting skeletal muscle relaxant, Neuropharmacology 14 (1975) 675-684.
11. M.L. Cotton and G. R. B. Down, Cyclobenzaprine, Anal. Profiles Drug Substances 17 (1988) 41.

12. F. Oesch, D.M. Jerina, and J. Daly, Substrate specificity of hepatic epoxide hydrase in microsomes and in a purified preparation: Evidence of homologous enzymes, Arch. Biochem. Biophys. 144 (1971) 253-261.

13. G. Belvedere, C. Pantarotto, V. Rovei, and A. Frigerio, Identification of Cyclobenzaprine-10, 11-Epoxide and other Metabolites after Incubation of Cyclobenzaprine with Rat Liver Microsomes, Xenobiotica 5 (1975) 765-771.

14. G. Belvedere, V. Rovei, C. Pantarotto, and A. Frigerio (Eds), Advances in Mass Spectrometry in Biochemistry and Medicine, Volume II, Spectrum Publications, Inc., New York, 1975.

15. G. Belvedere, C. Pantarotto, V. Rovei, and A. Frigerio, Identification of 10, 11-epoxide and other cyclobenzaprine metabolites isolated from rat urine, J. Pharm. Sci. 65 (1976) 815-821.

16. H.B. Hucker, S.C. Stauffer, A.J. Balletto, S.D. White, A.G. Zacchei, and B.H. Arison, Physiological disposition and metabolism of cyclobenzaprine in the rat, dog, rhesus monkey, and man, *Drug Metab. Dispos.* 3 (1978) 659-672.

17. H.B. Hucker, A.J. Balletto, B.H. Arison, A.G. Zacchei, Metabolism of cyclobenzaprine in the dog, Drug Metab. Dispos. 6 (1978) 184-192.

18. M. Constanzer, C. Chavez, B. Matuszewski, Development and comparison of high-performance liquid chromatographic methods with tandem mass spectrometric and ultraviolet absorbance detection for the determination of cyclobenzaprine in human plasma and urine, J. Chromatogr. B 666 (1995) 117-126.

19. D. Zhang, F.E. Evans, J.P. Freeman, Y. Yang, J. Deck, C.E. Cerniglia, Formation of mammalian metabolites of cyclobenzaprine by the fungus, Cunninghamella elegans, Chemico-Biological Interactions 102 (1996) 79-92.
20. M.V.B. Rao, B.C.K. Reddy, T.S. Rao, M Sivanadh, Development and validation of dissolution test for cyclobenzaprine hydrochloride pellets, Res. J. Pharm. Bio. Chem. Sci. 1 (2010) 315-319.
21. N.K. Ramadan, H.E. ZaaZaa, H.A. Mercy, Microsized graphite sensors for potentiometric determination of cyclobenzaprine hydrochloride in pure powder, tablets, and plasma J AOAC Int. 94 (2011) 1807-1814.
22. Y. Xiang, L. Zhou, Z. Qian, K. Peng, D. Li, X. Chen, H. Jianga and H. Zheng, Determination of cyclobenzaprine in human plasma by liquid chromatography-electrospray ionization tandem mass spectrometry and its application in a pharmacokinetic study, Biomed. Chromatogr. 26 (2012) 1083–1088.
23. J.-C. Li, F.-H. Chen, J.-D. Zhang, H.-J. Dong and S. Gao, A sensitive, fast and accurate liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–MS/MS) method for the pharmacokinetic study of cyclobenzaprine tablets, Afr J Pharm Pharmaco 6 (2012), 708-716.
24. ICH Harmonised Tripartite Guideline Q1B Stability Testing: Photostability Testing of New Drug Substances and Products, November 1996.
25. J.A. Ciaccio, Diastereospecific synthesis of an epoxide: an introductory experiment in organic synthetic and mechanistic chemistry, J. Chem. Educ., 1995, 72, 1037-1039.

Figure legends:

Figure 1. **The total ion chromatogram of cyclobenzaprine in an acidic aqueous solution using LC-APCI-MS.** Chromatographic conditions: column: Phenomenex Kinetex XB-C18 (100 x 4.6 mm, 2.6 μm); column temperature: 25^{o}C; flow rate: 1.0 mL min^{-1}; mobile phases: A: 0.1% formic acid in water, B: acetonitrile; gradient: 0-8 minutes from 20%-30% B, 8-18 minutes from 30%-40% B, 18-25 minutes from 40-50% B, 25-26 minutes from 50-20% B; column equilibration time: 10 min; injection volume: 10 μL; The optimal APCI-MS/MS parameters were as follows: Source temperature was 450^{o}C; the flow rates for curtain gas and collision gas were set at 15 and 6 psi, respectively. The nebulizer current was at 2.0 μA. Declustering potential, collision energy, entrance potential and collision cell exit potential were set at 100 V, 23 eV, 10 V and 13 V, respectively, for cyclobenzaprine and its degradants. Dwell Time was set at 100 ms. Mass range: 120-400 amu.

Figure 2. Proposed fragmentation and tandem mass spectrum of Impurity XII. Chromatographic conditions are the same as in Figure 1. Basic settings of the mass spectrometer are the same as in Figure 1 except MS/MS conditions: collision energy: 23 eV; mass range: 50-400 amu.

Figure 3. Proposed fragmentation and tandem mass spectrum of Degradant I. Chromatographic and mass spectrometric conditions are the same as in Figure 2.

Figure 4. Proposed fragmentation and tandem mass spectrum of Degradants II and VII. Chromatographic and mass spectrometric conditions are the same as in Figure 2.

Figure 5. Proposed fragmentation and tandem mass spectrum of Degradant IV. Chromatographic and mass spectrometric conditions are the same as in Figure 2.

Figure 6. Proposed fragmentation and tandem mass spectrum of Degradant VI. Chromatographic and mass spectrometric conditions are the same as in Figure 2.

Figure 7. Proposed fragmentation and tandem mass spectrum of Degradants VIII and X. Chromatographic and mass spectrometric conditions are the same as in Figure 2.

Figure 8. Proposed fragmentation and tandem mass spectrum of Degradants IX and XI. Chromatographic and mass spectrometric conditions are the same as in Figure 2.

Figure 9. Proposed fragmentation and tandem mass spectrum of Degradant XII. Chromatographic and mass spectrometric conditions are the same as in Figure 2.

Figure 10. Proposed degradation pathways of cyclobenzaprine.

Table 1. Major degradants identified during the preliminary forced degradation studies.

Study ID	Stress Conditions	Number of Major Degradants Identified
Acid stressing	Stress the drug solution for 6 hours at 60^{o}C in the solution of 1 N HCl	0
Base stressing	Stress the drug solution for 6 hours at 60^{o}C in the solution of NaOH	0
Peroxide stressing	Stress the drug solution for 12 hours at room temperature in 3% H_2O_2	1 (Degradant XII)
Heat/Humidity stressing	Stress the drug powder for 6 months under a high temperature of 40^{o}C and relative humidity (RH) of 75%	1 (Degradant II)
Light stressing	Stress the drug powder under the UV-visible light conditions stated in the option 2 of the ICH photostability guidelines	1 (Degradant XIII)

Table 2. UV and LC-MS/MS data of cyclobenzaprine and its degradants.

ID	MW	Mass Change	Major LC-MS/MS Fragments	UV (λ_{max}, nm）
CBA	275	-	276, 231, 216, 205, 191, 153, 115, 84, 58	225, 245, 290
I	339	+64	340, 322, 304, 277, 259, 231, 221, 207, 193, 179, 58	240
II	309	+34	310, 292, 264, 247, 229, 219, 203, 191, 179, 91, 84, 58	215, 245
III	309	+34	310, 292, 264, 247, 229, 219, 203, 191, 179, 91, 84, 58	215 245
IV	325	+50	326, 308, 280, 257, 245, 229, 219, 202, 191, 179, 131, 84, 58	214, 240
V	325	+50	326, 308, 280, 257,245, 229,219, 202, 191, 179, 131, 84, 58	214, 240
VI	307	+32	308, 290, 245, 229, 217, 205, 191, 131, 91, 72, 58	235, 260
VII	309	+34	310, 292, 247, 229, 221, 203, 193, 179, 131, 72, 58	215, 230, 290
VIII	291	+16	292, 274, 247, 229, 219, 203, 191, 179, 91, 84, 58	224, 255
IX	291	+16	292, 264, 247, 233, 219, 207, 191, 178, 155, 119, 91, 84, 58	215, 240
X	291	+16	292, 274, 247, 229, 219, 203, 191, 179, 91, 84, 58	225, 245
XI	291	+16	292, 247, 229, 221, 203, 193, 178, 115, 72, 58	290
XII	291	+16	292, 274, 262, 231, 216, 191, 178, 115, 100, 74, 58	225, 245, 290

Fig 1

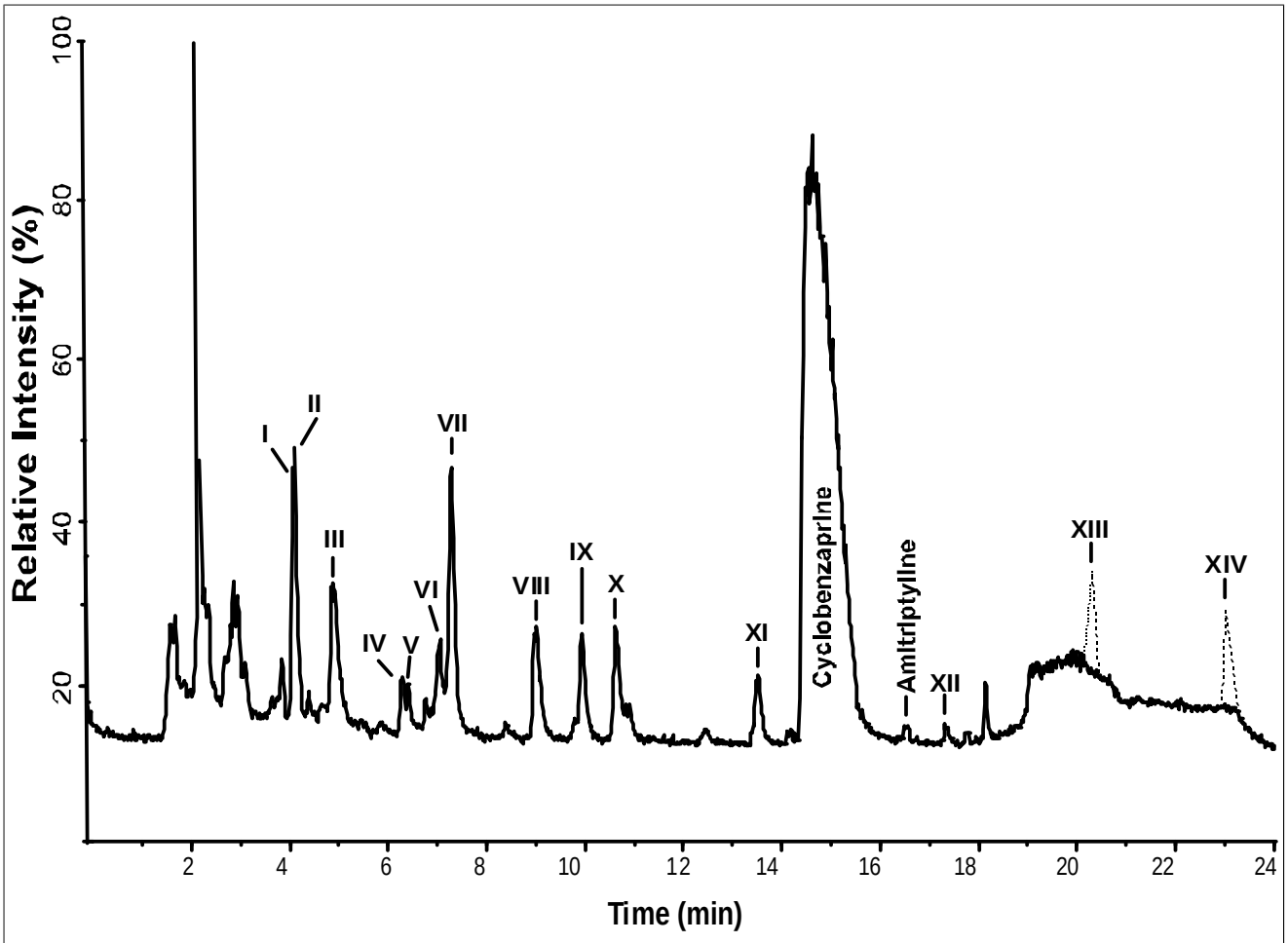
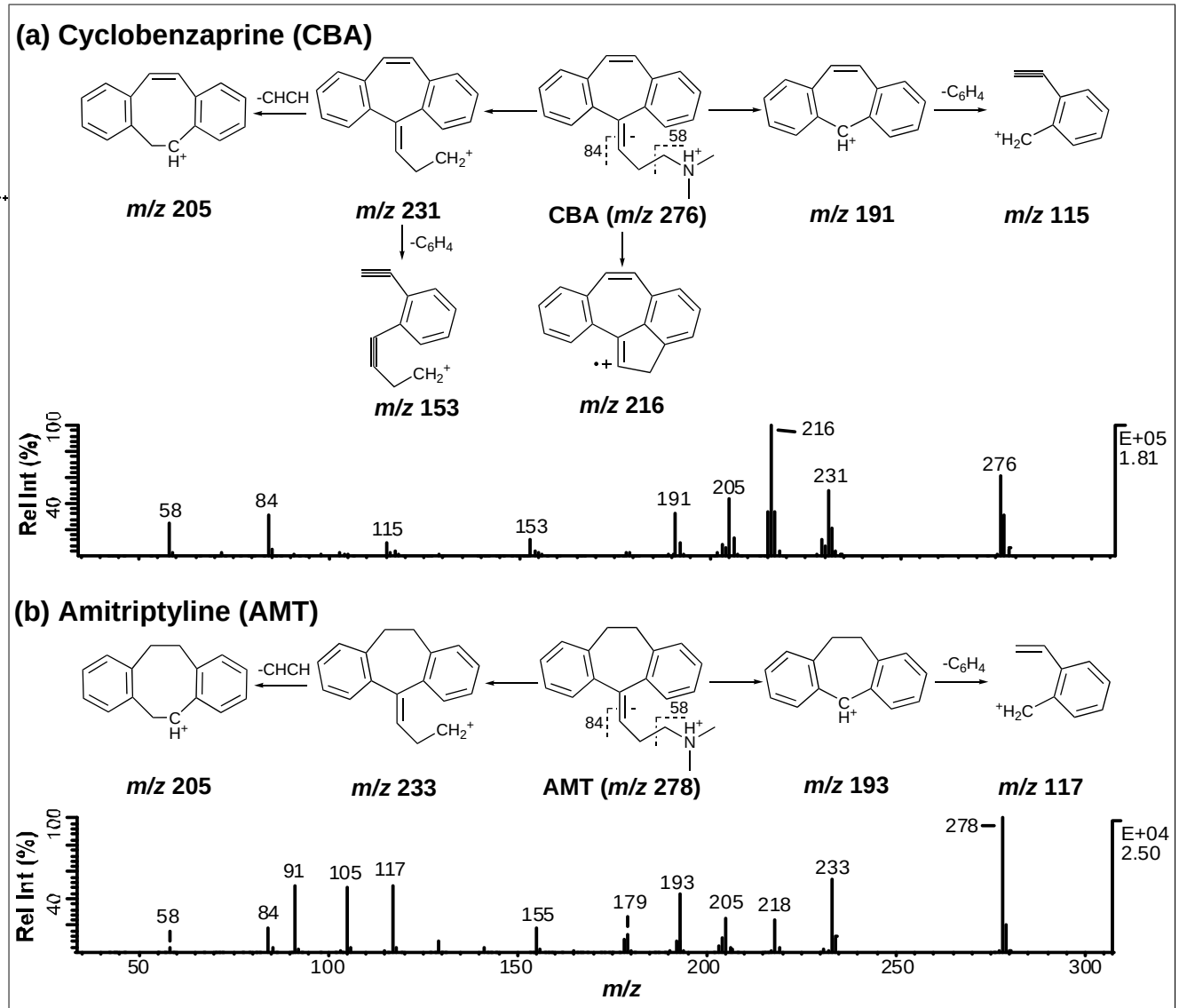


Fig 2



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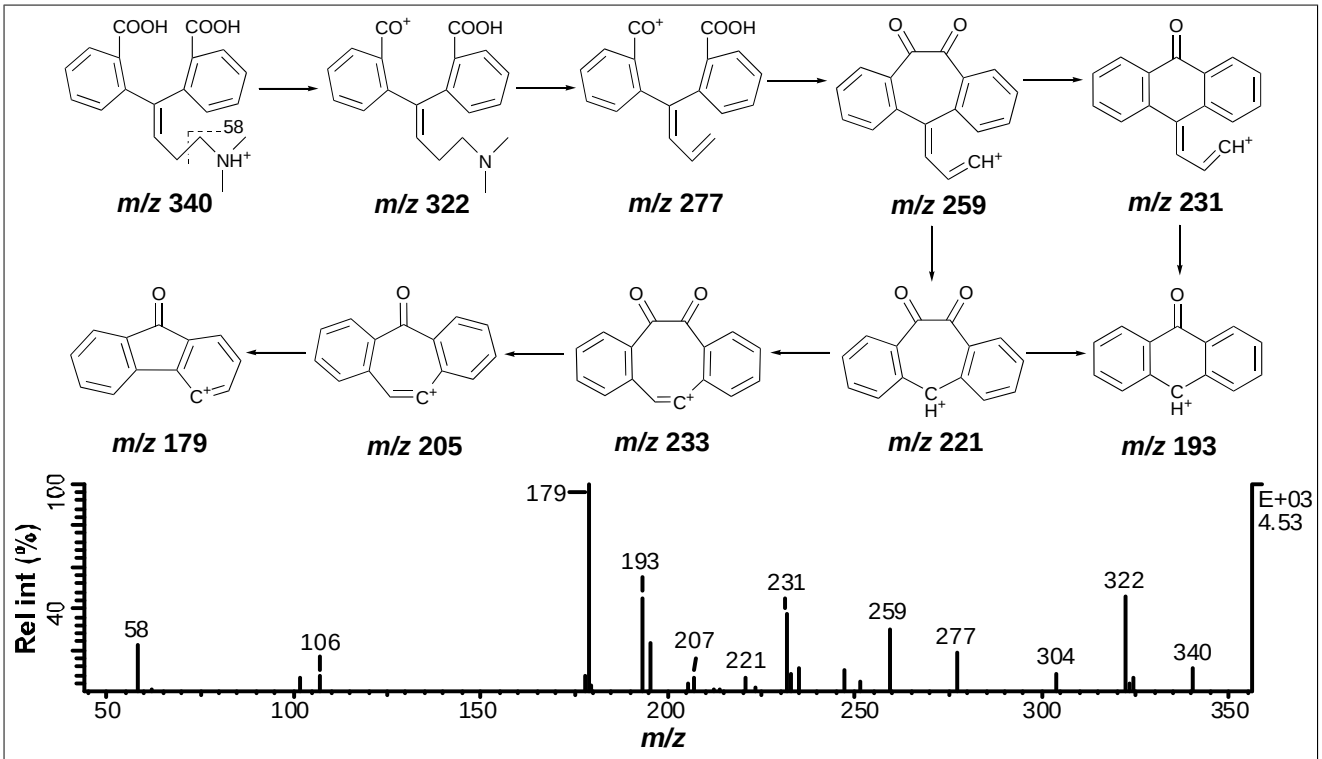

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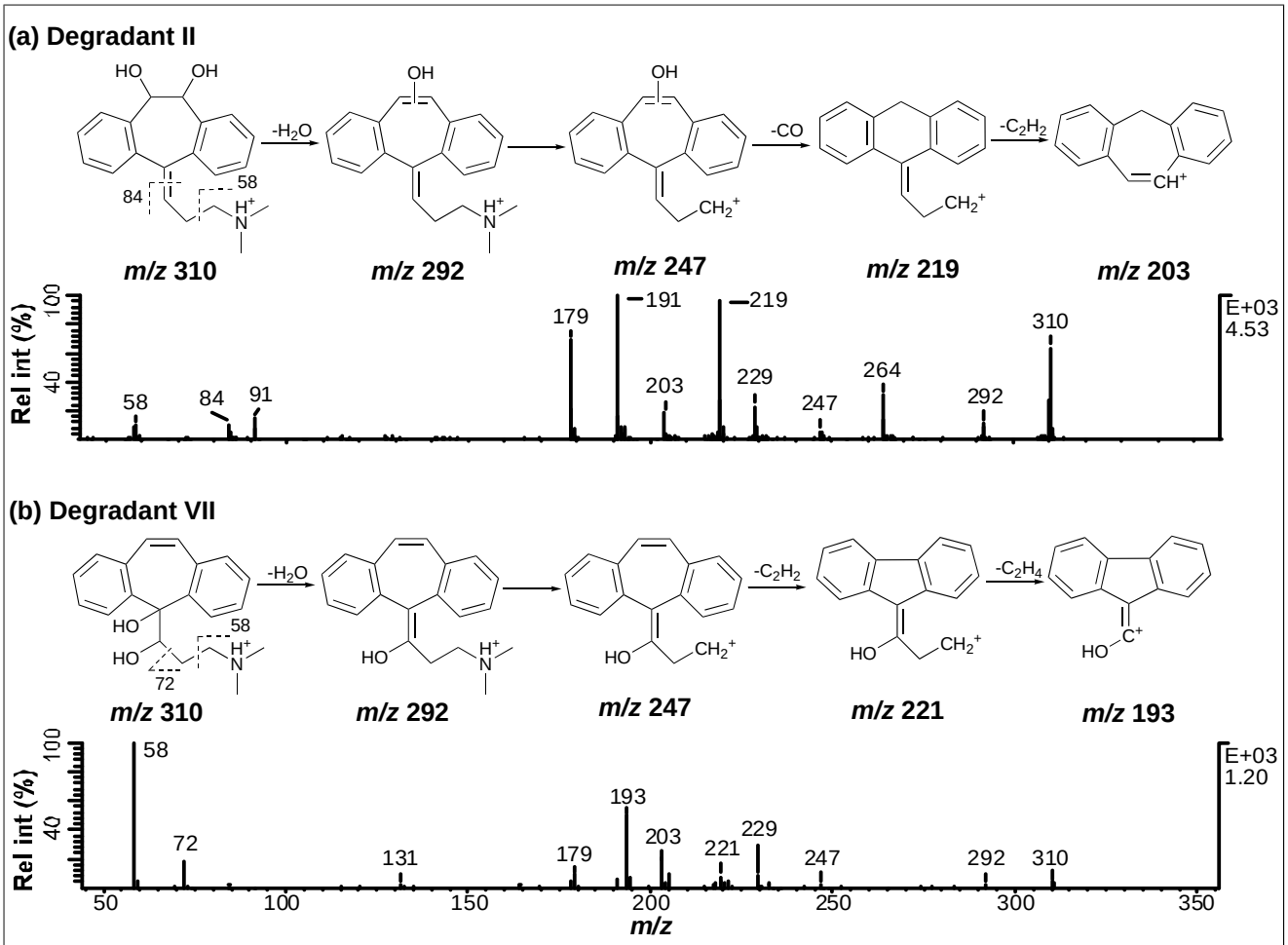

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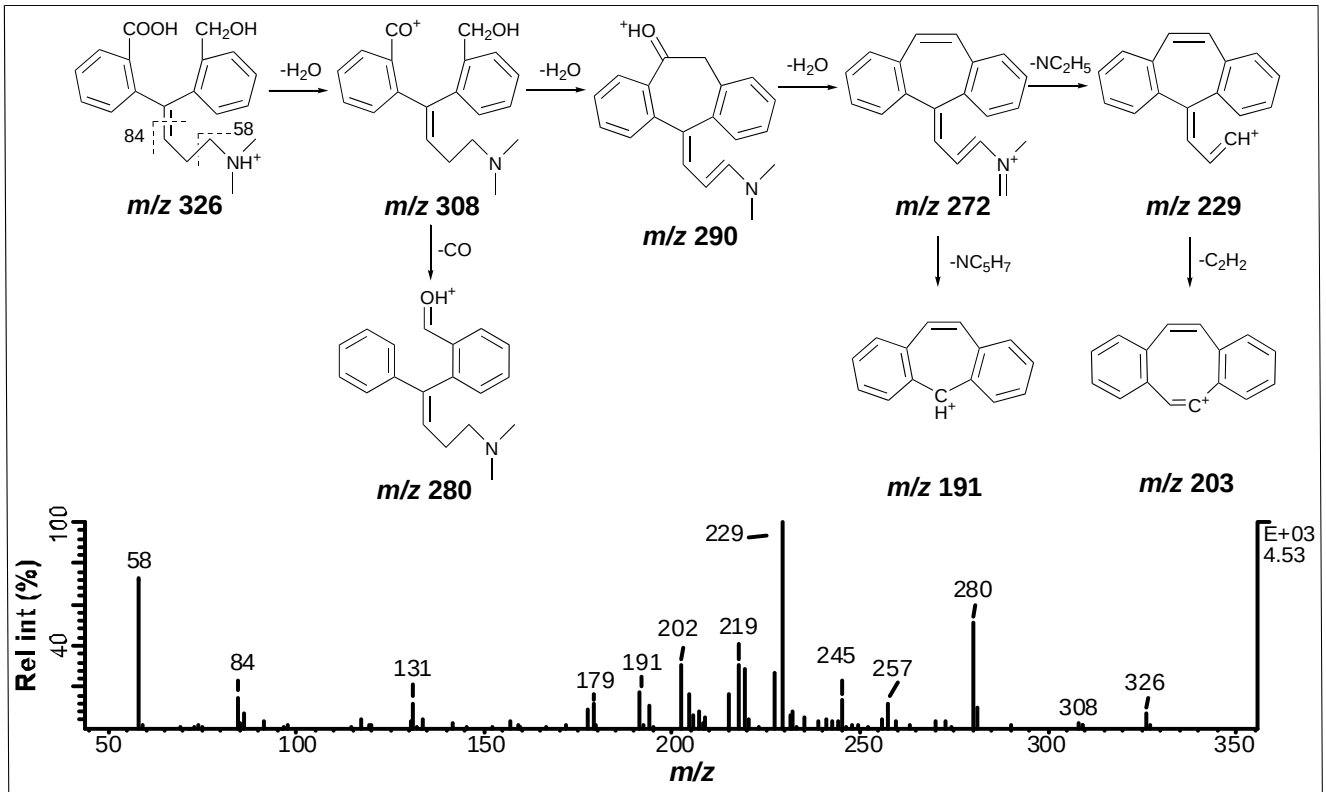

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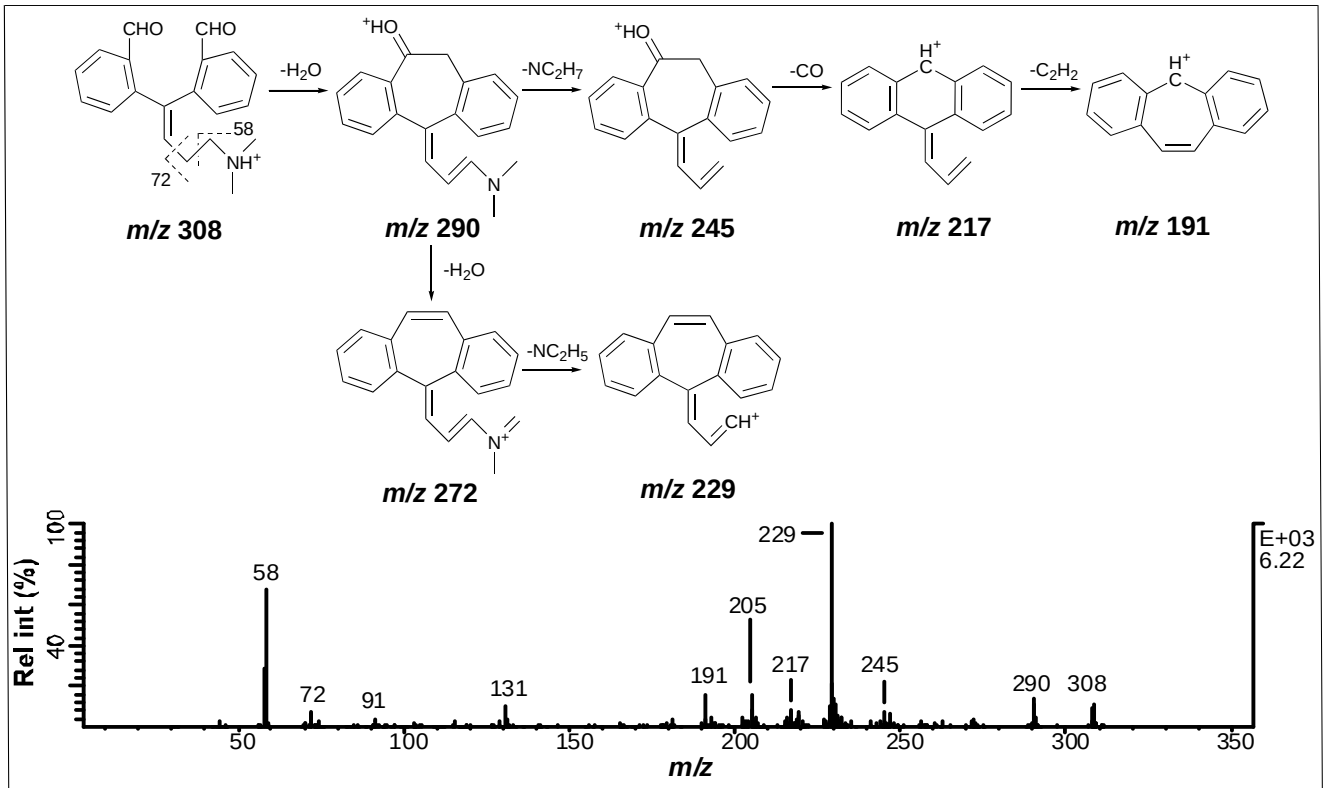

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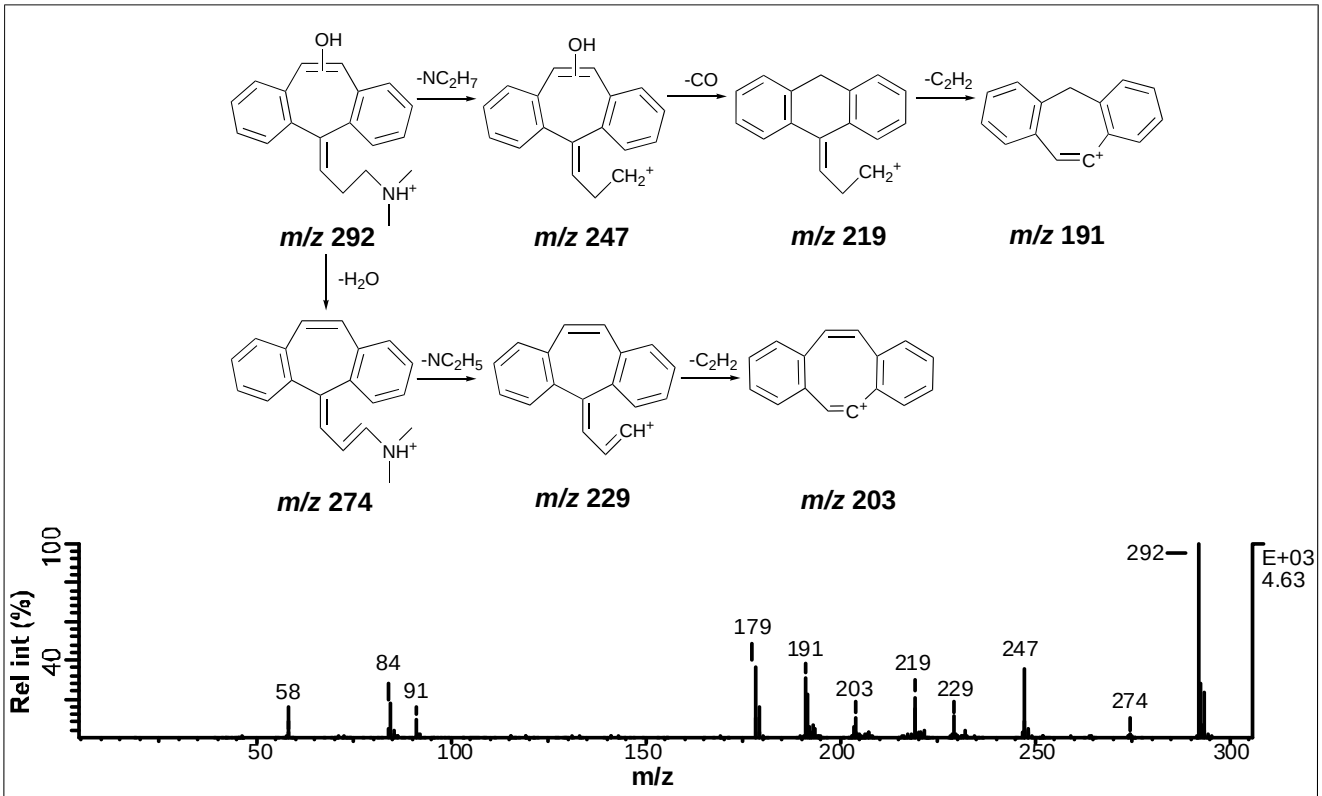

Fig 8

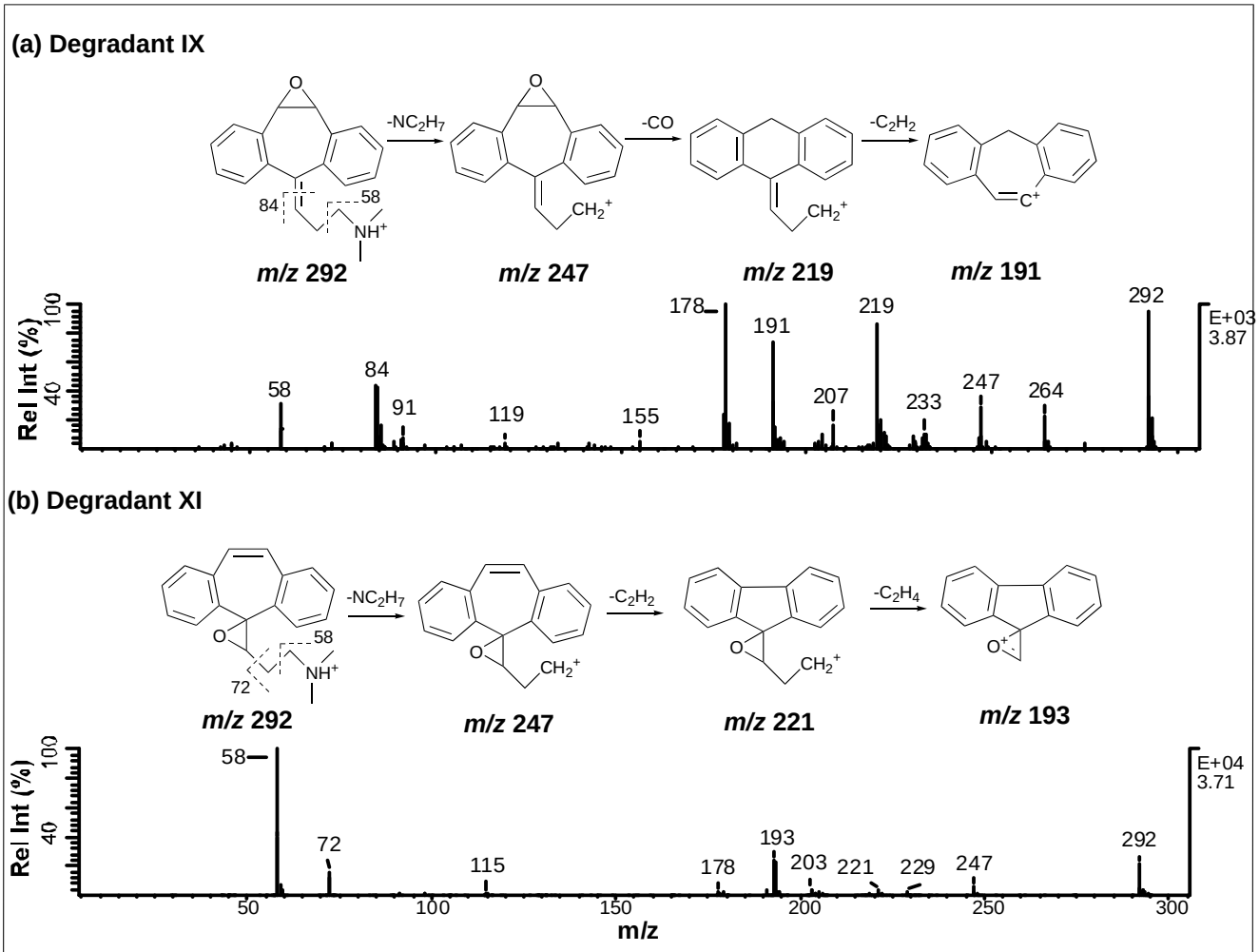

Fig 9

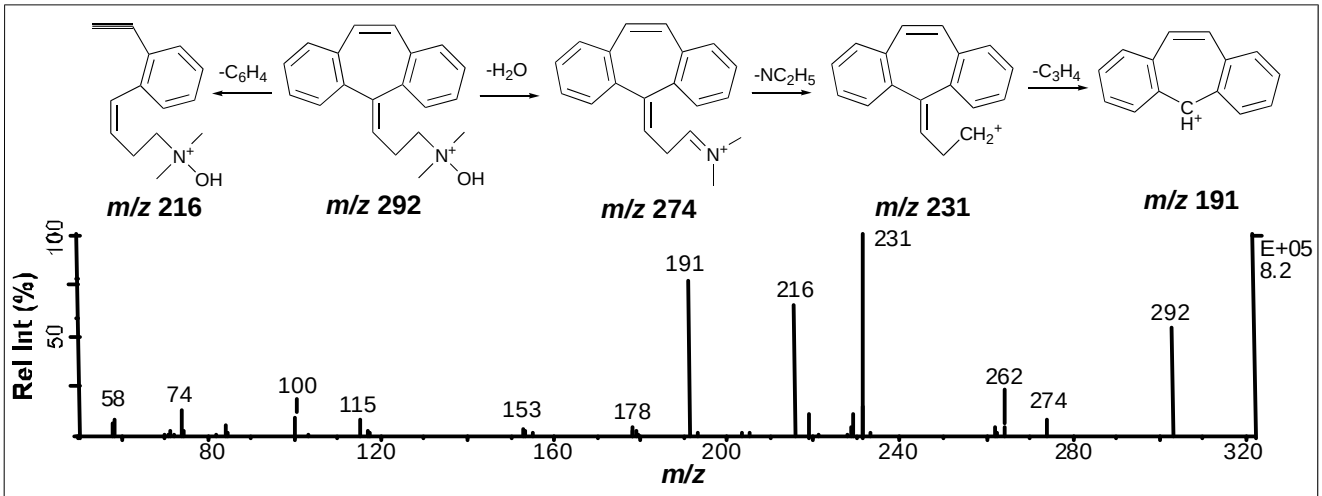

Fig 10