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Characterization of degradation products of midazolam maleate by UHPLC-HR-IT-MS^n and NMR

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Forced degradation studies on midazolam maleate were carried out according to ICH guidelines. Midazolam maleate was subjected to acidic and basic hydrolysis, oxidation, photolysis, high humidity and thermal stress conditions, and the resulting degradation products were investigated by HPLC. Significant degradation of the drug was observed under acidic/basic hydrolysis and thermal stress conditions. The thermal degradation product (Impurity I) was isolated using column chromatography and its structure was elucidated by UHPLC-HR-IT-MS^n and extensive NMR studies, which was not reported in previous literatures. The acidic/basic hydrolytic degradation product (Impurity II) was characterized by UHPLC-HR-IT-MS^n technique and previous literature. The fragmentation pathways of these two degradation products are also described in the paper.

1. Introduction

Midazolam maleate (MDM, Fig. 1), a derivative of benzodiazepine drugs, is a strong sedative with significant sedating, muscle relaxation, anticonvulsant and anxiolytic effects. It is used as a premedication and an inducer of general anesthesia (Ashton 1994; Dornauer and Aston 1983) in surgery.

MDM has not been embodied in the United States Pharmacopoeia (USP), European Pharmacopoeia (EP) or Chinese Pharmacopoeia (ChP), but midazolam is recorded in these Pharmacopoeias. ChP is the only one which records midazolam maleate tablets (ChP, 2015). In the available monographs, ten related impurities of midazolam are listed in EP (EP 8.0, 2014), and eight are listed in USP (USP 36, 2013). However, no related substances or impurities of MDM are mentioned. Literature reports were concentrate on the clinical application and pharmacological fields (Ren et al. 2007; Blackmon et al. 1984; Reves et al. 1979), and pay less attention to the related substances of MDM (Huang et al. 2010, 2011). However, the ICH guidelines Q3A and Q3B (ICH, 2006) require the characterization of actual and potential impurities (process and degradation related) in a drug substance or product. These characterizations can be facilitated by forced degradation tests of the drug under various conditions including hydrolysis, oxidation, humidity, dry heat and photolysis (ICH Q1A, 2003). Comprehensive details on the degradation behavior of drugs can help to maintain their quality and pharmaceutical safety (Maggio et al. 2013; Singh et al. 2012).

The purpose of this study was to characterize degradation products of MDM under various stress conditions. The forced degradation tests were conducted and yielded two major degradation products (named Impurity I and Impurity II), which were characterized by ultra high performance liquid chromatography/high resolution ion trap mass spectrometry (UHPLC-HR-IT-MS^n). Impurity I was isolated by column chromatography, and 1D (^{1}H, ^{13}C, DEPT) and 2D (^{1}H-^{1}H COSY, HSQC, HMBC) NMR studies were performed to confirm its structure, which was an addition product of midazolam and maleic acid and was not reported in previous literatures. Impurity II was a ring-opening hydrolysis product of midazolam, characterized by UHPLC-HR-IT-MS^n and previous literature. The fragmentation pathways of these two degradation products are also described in this paper. Our findings are very important for the quality control of MDM and helpful to improve relevant standards.

2. Investigations, results and discussion

2.1. HPLC evaluation of the degradation samples

The degradation behaviors of MDM under various forced degradation conditions were studied using the method described in Section 3.2. The results are summarized in Table 1. The overlay of HPLC chromatograms of all stress degradation samples is given in Fig. 2. Significant degradation of the drug was observed under acidic and basic hydrolysis and thermal conditions, whereas it was stable under oxidative, high humidity and photolytic conditions. The impurity formed under the thermal condition was named Impurity I. The impurities formed in acidic and basic hydrolytic conditions had the same retention time, same quasi-molecular ion and fragment ions detected by MS. Therefore they were the same compound (named Impurity II).

2.2. Characterization of Impurity I

This degradation product was formed under thermal conditions and had an experimental accurate *m/z* of 442.09592 (Fig. 3 a), which was the same as MDM. The $[M+H]^+$ ion fragmented in mass studies to form product ions of *m/z* 424 (loss of H_2O, I-1), *m/z* 407 (loss of Cl, I-2), *m/z* 398 (loss of CO_2, I-3), *m/z* 325 (loss of $C_4H_5O_4$, I-4) and *m/z* 309 (loss of $C_4H_7NO_4$, I-5) in MS^2 (Fig. 3 b). In these fragments, I-3 and I-4 displayed high abundance, and MS^3 analyses were further conducted on the ions *m/z* 398 and *m/z* 325, respectively. Besides *m/z* 363 (loss of Cl, I-3-1), *m/z* 325 (I-4) and *m/z* 309 (I-5), the former also generated *m/z* 290 (loss of $C_3H_5ClO_2$, I-3-2) and *m/z* 244 (loss of $C_7H_{10}N_2O_2$, I-3-3) (Fig. 3 c); besides *m/z* 290 (-Cl, I-4-1, i.e. I-3-2), the latter mainly generated *m/z* 257 (loss of $C_3H_4N_2$, I-4-2) (Fig. 3 d). The errors between the accurate mass measurements and theoretical mass for the protonated molecular ions and fragmentation ions were all below ±5 ppm.

Based on the above data the molecular weight of Impurity I was 441. More importantly, the $[M+H]^+$ ion produced a product ion of *m/z* 325 (midazolam radical) in MS^2, suggesting that the structure of midazolam still existed in Impurity I. Its structure should consist of midazolam and maleic acid, that is, the maleic acid connects directly to midazolam. However, the linkage location of the two molecules and detailed structure of Impurity I had to be clarified by NMR experiments.

1D and 2D NMR spectra of isolated Impurity I, including ^{1}H NMR, ^{13}C NMR, DEPT-135, ^{1}H-^{1}H COSY, HSQC and HMBC, were recorded. Meanwhile, the ^{1}H NMR spectrum of MDM was deter-

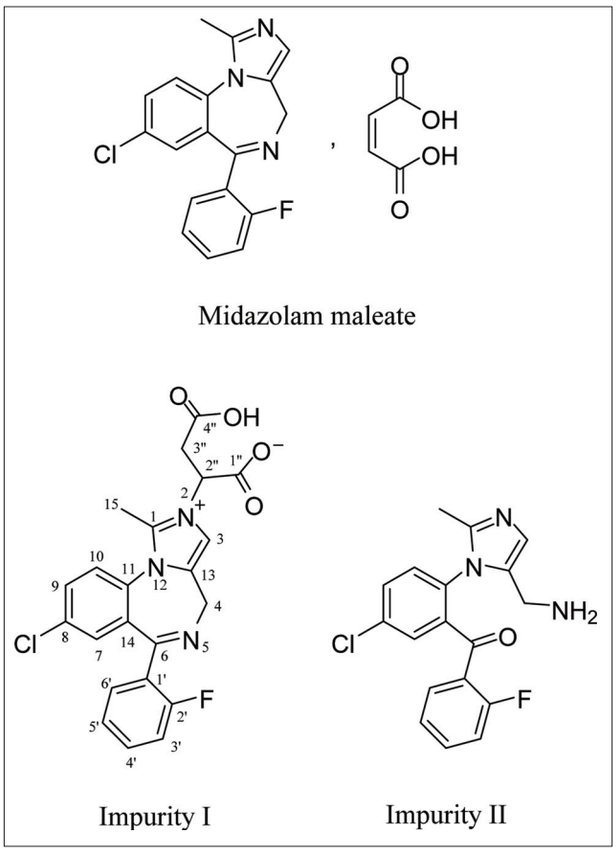


Fig.1: Structures of midazolam maleate (MDM), Impurity I and Impurity II

mined for comparison. The structure deduction of Impurity I was as follows. (1) ^{1}H NMR spectra (Fig. 4) indicated that the methyl still existed in comparison to MDM. The number of aromatic proton signals was also the same as observed for midazolam, namely eight. The signals of methylene on position 4 could be still observed, which suggested the existence of seven-membered-ring. However, the peak of δ 6.17 ppm (the alkenyl hydrogen of maleic acid in MDM, 2H) was not observed, three other peaks (δ 2.86, 3.36 and 5.25 ppm, 3H) arose instead. This suggested that the double bond of maleic acid had been destroyed, namely, alkenyl turned to methine and methylene. (2) DEPT-135 showed that two methylenes existed in Impurity I, which was one more than that of MDM, confirming that the alkenyl in maleic acid turned to methine and methylene. (3) In HMBC (Fig. 5), a correlation was found between H-2'' and C-1, which suggested that C-2'' in maleic acid was connected with position 2 (N atom) in midazolam. (4) In ^{1}H NMR spectrum of Impurity I, the signal of H-3 (δ 7.92 ppm) appeared at lower field than that of midazolam (δ 6.96 ppm) (Tao 2012) due to the deshielding effect as a result of position 2 connected with maleic acid.

On the basis of the MS^n and the NMR data, we conclude that Impurity I is an addition product of midazolam and maleic acid (Fig. 1). The NMR data are listed in Table 2. The fragmentation pathway of Impurity I is shown in Scheme 1. Since the chlorine atom is directly connected to the benzene ring as aromatic chlorides, it is lost in a heterolytic cleavage way of neutral radical as the literature reported (Chen and Tu 2001), showing strong peaks of $[M+H-Cl]^+$, not $[M+H-HCl]^+$. The reaction mechanism of Impurity I probably is nucleophilic addition. The olefinic carbon electron density is decreased owing to the electron withdrawing action of carboxyl groups in maleic acid. In this reaction, midazolam was a nucleophilic reagent due to N atom with lone pair electrons. N atom of midazolam attacked the olefinic carbon of maleic acid, resulting in the addition product of Impurity I.

2.3. Characterization of Impurity II

Impurity II was formed under acidic and basic hydrolytic conditions. The UHPLC-ESI-IT-MS^n analysis indicated that the $[M+H]^+$ ion of Impurity II was 344, which increased 18 Da compared with midazolam, suggesting that Impurity II was the hydrolysis product of midazolam. It was probably the open-ring form of midazolam reported previously (Andersin 1991) as shown in Fig. 1. In order to confirm this structure, the MS^n spectra of Impurity II were examined. The $[M+H]^+$ ion was reduced to product ions of *m/z* 327 (loss of NH_2, II-1), *m/z* 315 (loss of $CHNH_2$, II-2) and *m/z* 109 ($C_7H_6F^+$, II-3) in MS^2 (Fig. 6 a). MS^3 analysis was carried out on the ion *m/z* 315 (Fig. 6 b), and it produced fragments as follows: *m/z* 297 (loss of H_2O, II-2-1), *m/z* 274 (loss of $CHCH_2N$, II-2-2) and *m/z* 123 ($C_7H_4FO^+$, II-2-3). The errors between the accurate mass measurements and theoretical mass for the protonated molecular ions and fragmentation ions were all below ±5 ppm. The fragmentation pathway of Impurity II is shown in Scheme 2.

On the basis of the MS^n data and literature, Impurity II actually is a ring-opening hydrolysis product of midazolam. According to the literature, midazolam is in equilibrium with the open-ring form in acidic media. In our experiment, it was found that the open-ring form was generated not only in acidic media but as well in basic media.

3. Experimental

3.1. Samples

MDM was provided by Hubei Humanwell Pharmaceutical Co., Ltd. (Yichang, China). Methanol (HPLC grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate and acetic acid (HPLC grade) were purchased from Kermel (Tianjin, China). Ultra-pure water was obtained from a Millipore Milli-Q system (Bedford, MA, USA). Analytical reagent (AR) grade sodium hydroxide (NaOH), hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2, 30%) were purchased from Guangdong Guanghua Sci-Tech Co., Ltd. (Guangzhou, China).

3.2. Forced degradation studies

These studies were carried out under acidic and basic hydrolysis, oxidation, photolysis, high humidity and thermal stress conditions. For all the stress studies, the concentration of MDM was 0.5 mg·mL^{-1}. Acidic and alkaline hydrolytic studies were carried out in 1 mol·L^{-1} HCl for 2 h and 1 mol·L^{-1} NaOH for 1 h at 80 °C, respectively. For the oxidative

Table 1: Stress degradation for MDM

Stress conditions	Concentration of stressor	Exposed conditions	Duration	Degradation (%)[a]	
				Impurity I	Impurity II
MDM	-	-	-	0.027	0.015
Acid	1 mol·L^{-1} HCl	80 °C	2h	0.017	**1.606**
Base	1 mol·L^{-1} NaOH	80 °C	1h	0.337	**1.243**
Thermal	-	130 °C	2h	**4.685**	0.026
Oxidation	30% H_2O_2	RT[b]	24h	0.041	0.016
Humidity	92.5% RH	25 °C	5 days	0.040	0.018
Photolysis	4500 ± 500 lx	RT	5 days	0.030	0.016

a) Calculated by area percentage method (except blank peaks). *b*) RT, room temperature.

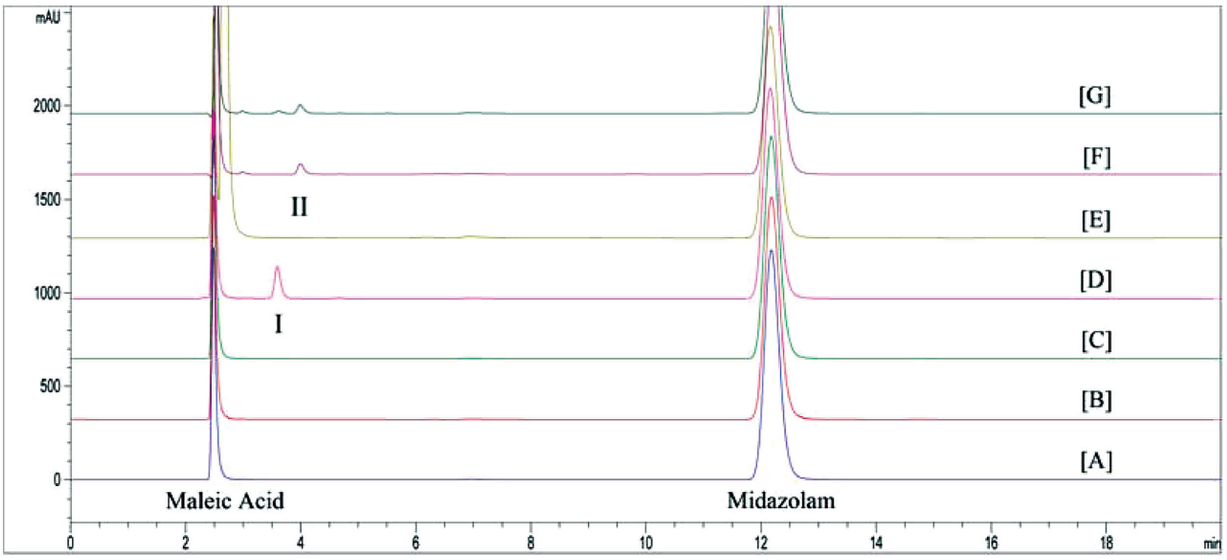


Fig.2: Chromatograms showing the separation of MDM and its degradation products
[A]. MDM, [B]. High humidity degradation, [C]. Photo degradation, [D]. Thermal degradation, [E]. Oxidative degradation, [F]. Acidic degradation, [G]. Basic degradation
I. Impurity I (3.56min) II. Impurity II (3.96min)

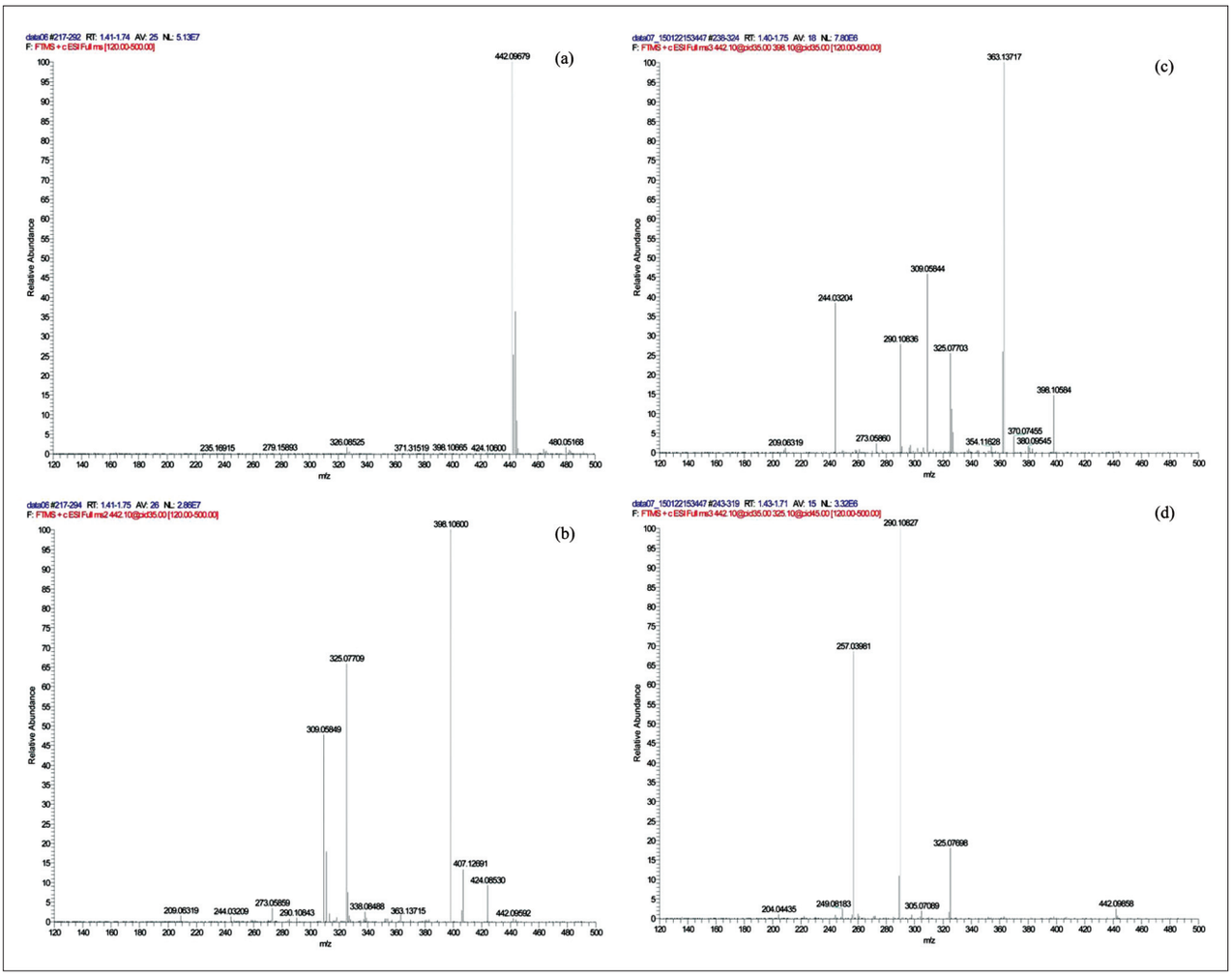


Fig.3: UHPLC-HR-IT-MS^n mass spectra of Impurity I
(a). MS (b). MS^2 (*m/z* 442) (c). MS^3 (*m/z* 398) (d). MS^3 (*m/z* 325)

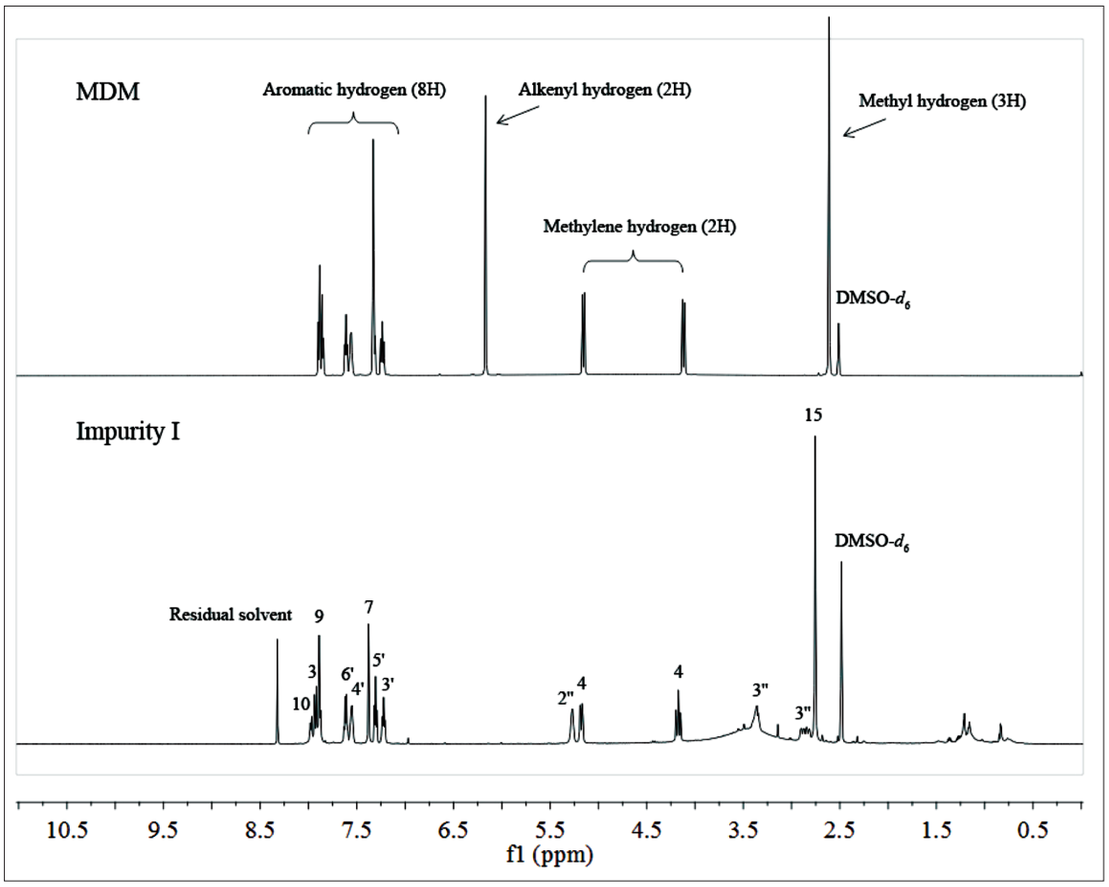


Fig.4: Overlaid ^{1}H NMR spectra of Impurity I and MDM in DMSO-d_6

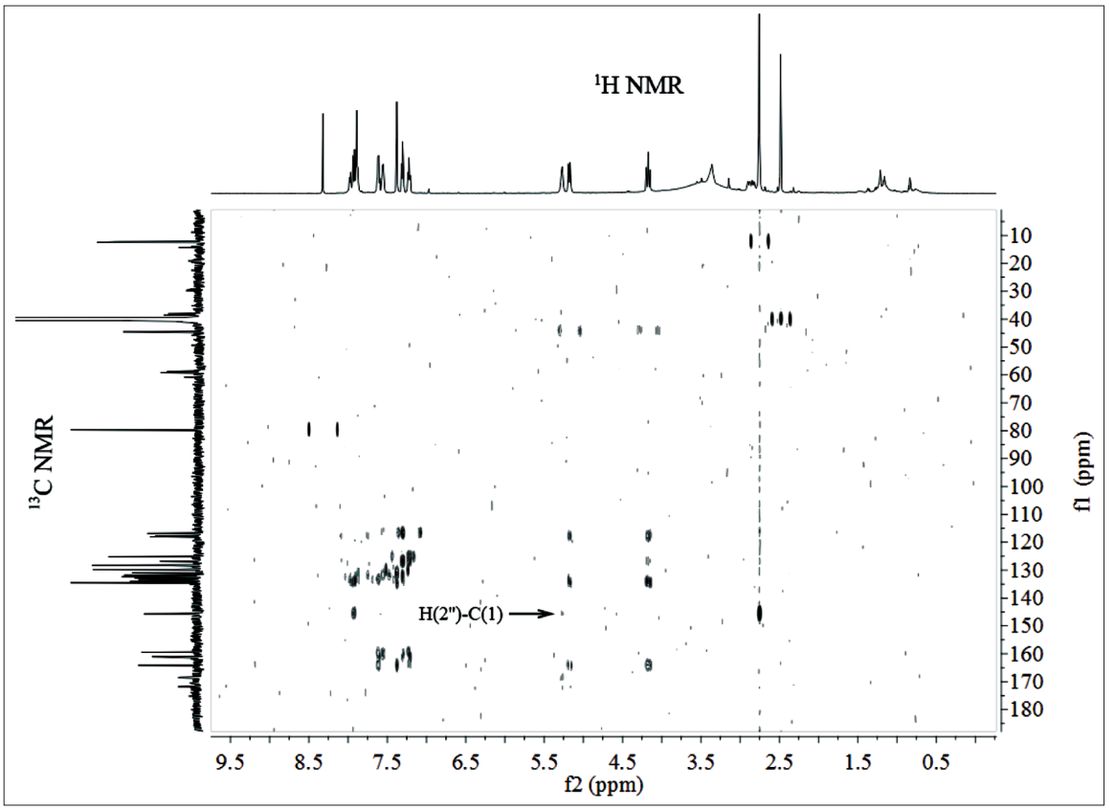


Fig.5: HMBC NMR spectrum of Impurity I in DMSO-d_6

Table 2: NMR chemical shifts assignment of Impurity I in DMVSO-d_6

Position	^{1}H (δ ppm)	^{13}C (δ ppm)	DEPT	^{1}H-^{1}H COSY	HMBC
1	-	145.63	-		-
3	7.92, overlapped	117.60	CH		C-1, C-13
4	4.15 (t), 5.15 (dd)	44.48	CH_2		C-3, C-6, C-13
6	-	164.10	-		-
7	7.37 (br s)	129.88	CH	H-9	C-6, C-8, C-9, C-14
8	-	134.59	-		-
9	7.88, overlapped	132.41	CH	H-7, H-10	C-8, C-14
10	7.97 (d)	128.34	CH	H-9	C-11, C-8
11	-	132.87	-		-
13	-	134.13	-		-
14	-	131.03	-		-
15	2.47 (s)	12.20	CH_3		C-1
1'	-	126.80	-		-
2'	-	159.53, 161.13	-		-
3'	7.21 (dt)	116.74	CH	H-4'	C-1', C-2', C-5'
4'	7.55 (m)	133.47	CH	H-3', H-5'	C-2', C-6'
5'	7.30 (br t)	125.23	CH	H-4', H-6'	C-1', C-3', C-4', C-6'
6'	7.61, overlapped	131.83	CH	H-5'	C-2', C-4', C-6
1''	-	168.60	-		-
2''	5.25 (m)	58.77	CH	H-3''	C-1, C-1''
3''	2.86 (m), 3.36 (m)	38.29	CH_2	H-2''	-
4''	-	171.71	-		-

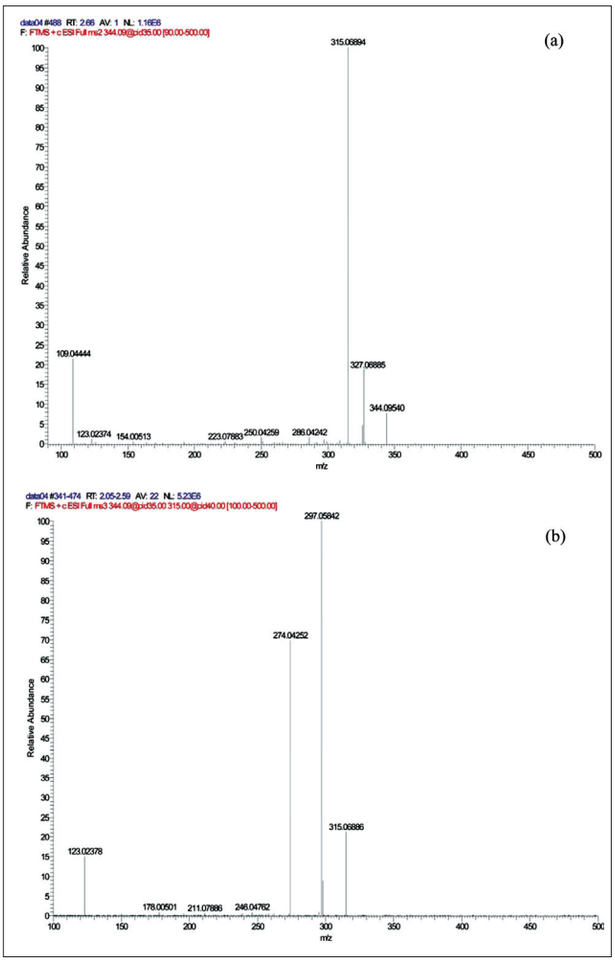


Fig.6: UHPLC-HR-IT-MS^n mass spectra of Impurity II (a). MS^2 (*m/z* 344) (b). MS^3 (*m/z* 315)

degradation, approximate 12.5 mg of MDM dispersed in 2 ml 30% H_2O_2 was placed in dark at room temperature for 24 h. The photodegradation study was carried out in a photostability chamber (YPZ220, GB/T 17263-1998, China) by exposing the drug in the form of a thin layer under 4500 ± 500 lx for 5 days, and the chamber was set at a temperature of 25 °C and RH of 55% during the experiment. The thermal stress testing was carried out by putting the drug in the form of a thin layer in an oven at 130 °C for 2 h. The high humidity degradation study was conducted by putting the drug in a dryer with saturated aqueous solution of KNO_3 (relative humidity 92.5%) at 25 °C for 5 days. All the degradation samples were neutralized and diluted with the mobile phase.

3.3. HPLC method

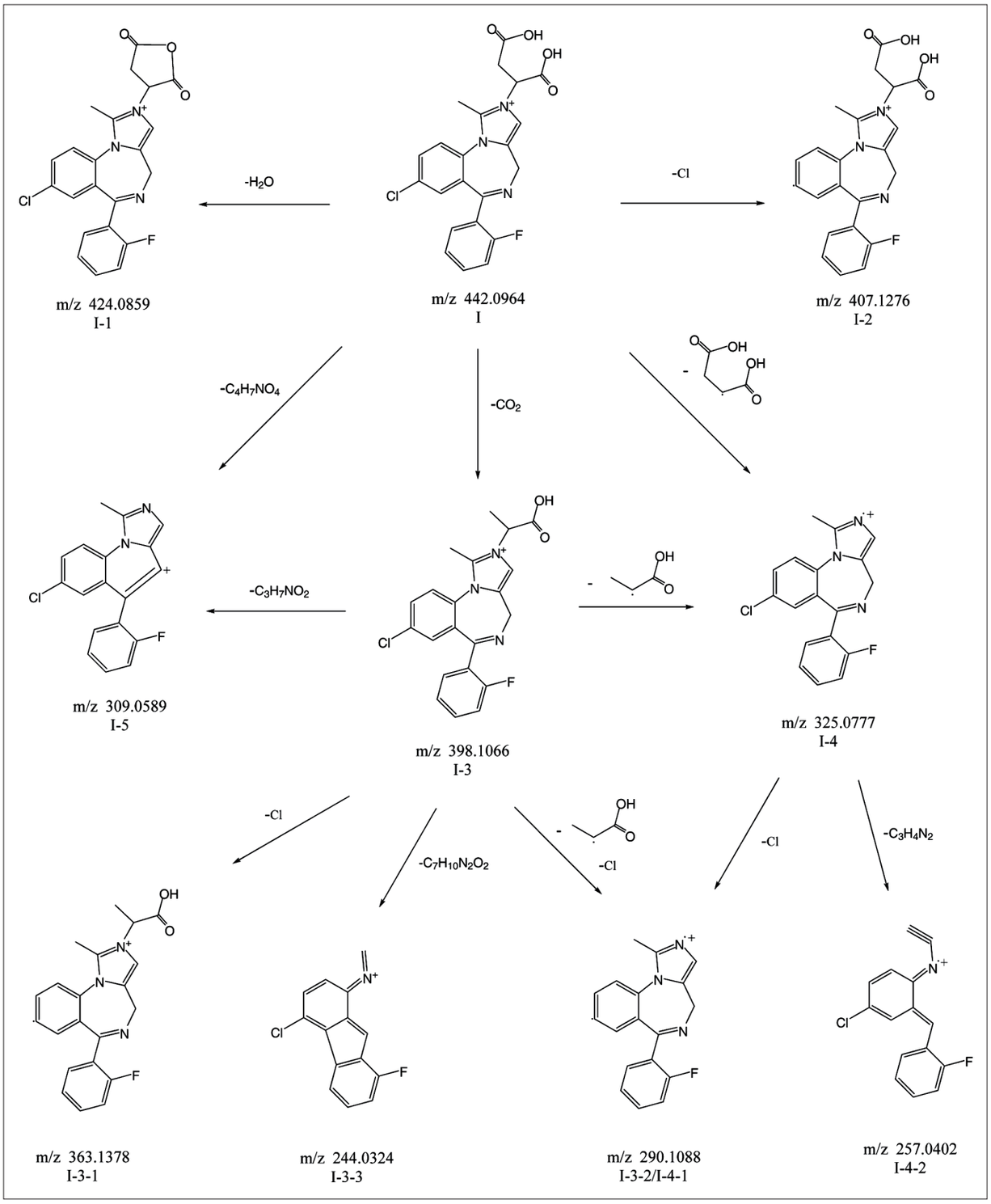
The forced degradation samples were analyzed on an Agilent 1260 Infinity HPLC system (Agilent Technologies Inc, CA, USA), which consisted of a binary pump, a column oven and a UV detector. The data were acquired and processed by Chemstation software (B0403). The drug and its degradation products were separated on an Inertsustain C_{18} column (250 mm × 4.6 mm i.d., 5 µm) at 30 °C using 220 nm as detection wavelength. A desirable separation was obtained with a mobile phase of 10 mmol·L^{-1} ammonium acetate (adjusting pH to 4.5 with acetic acid)-methanol (35:65, v/v), which was run at a flow rate of 1.0 mL·min^{-1}. The injection volume was 10 µL.

3.4. UHPLC-HR-IT-MSn st"dies on the degradation products

The UHPLC-HR-IT-MS^n system consisted of an UltiMate 3000 UHPLC system (Thermo Scientific, CA, USA) and a mass spectrometer (LTQ Orbitrap Elite, Thermo Scientific, MA, USA). The UHPLC system consisted of a pump, an auto-sampler, a column compartment and a PDA detector. The data were acquired and processed using Thermo Xcalibur software (version 2.2). In order to get a complete fragmentation pattern of the degradation products, MS^n studies were performed in an ESI positive mode. An UPLC™ BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 µm) was used for chromatographic separation at 30 °C. The mobile phase was duly optimized from HPLC analytical method by changing the proportion of water phase and organic phase to 45:55 (v/v). The flow rate was 0.25 mL·min^{-1} and the injection volume was 5 µL. UV detection was performed at 220 nm. Three stage mass spectra were recorded in ESI$^+$ mode using appropriate ionization potentials (35.0-40.0 eV). The operating conditions for recording MS scan were optimized as follows: mass range, *m/z* 120-500; capillary temperature, 350 °C; sheath gas flow rate, 30 arb; auxiliary gas flow rate, 5 arb; source voltage, 3.5 kV; source current, 100 µA; desolvation temperature , 200 °C; sweep gas flow rate, 1 arb.

3.5. Isolation of Impurity I

Impurity I was isolated from the thermal degradation sample with column chromatography. First acidified and dehydrated ethyl acetate (mixing 1 mol·L^{-1} HCl with the same volume of ethyl acetate, then extracting the organic layer and dehydrating with anhydrous sodium sulfate) - methanol (7:1, v/v) was used as eluent to remove midazolam and other impurities. Then the proportion of eluent was changed to 4:1 (v/v) for collecting the impurity. The collected eluent was detected by acidified and dehydrated ethyl acetate



Scheme 1 Mass fragmentation pathway of Impurity I (*m/z*: theoretical value)

- methanol (1:1, v/v). The collected impurity solution was evaporated to dryness under vacuum at 40 °C, then drained the residual solvent by oil pump to obtain Impurity I.

3.6. NMR spectroscopy

Nuclear magnetic resonance spectra were recorded on a Bruker Advance 600 MHz spectrometer (Bruker, Bremerhaven, Germany), using DMSO-d_6 solution with tetramethylsilane (TMS) as the internal standard.

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Conflicts of interest: None declared

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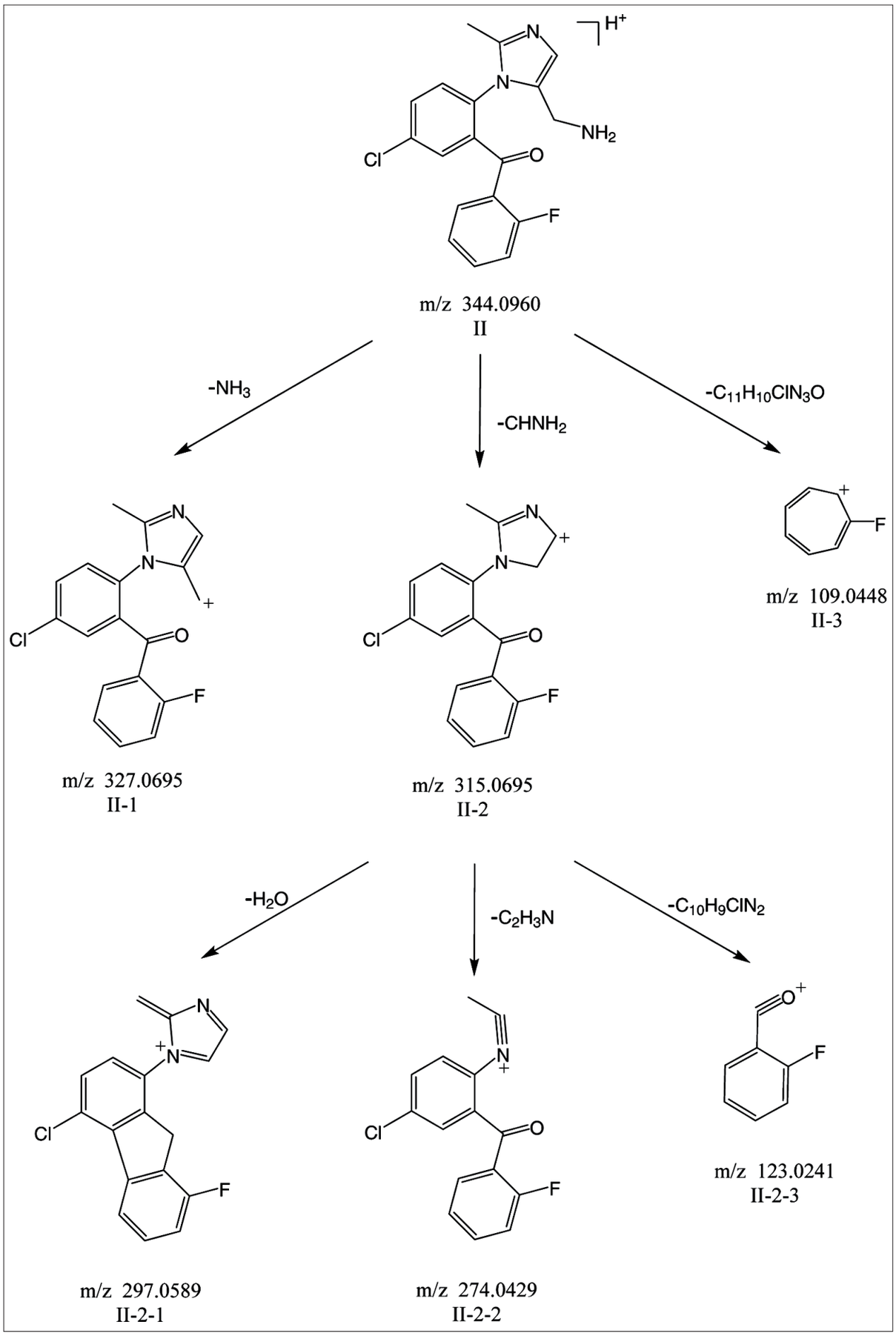

Scheme 2 Mass fragmentation pathway of Impurity II (*m/z*: theoretical value)

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