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ORIGINAL PAPER

An integrated method for degradation products detection and characterization using hybrid ion trap/time-of-flight mass spectrometry and data processing techniques: Application to study of the degradation products of danofloxacin under stressed conditions

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Abstract A new strategy using hybrid ion trap/time-of-flight mass spectrometry coupled with high-performance liquid chromatography and post-acquisition data mining techniques was developed and applied to the detection and characterization of degradation products of danofloxacin. The degradation products formed under different forced conditions were separated using an ODS-C18 column with gradient elution. Accurate full-scan MS data were acquired in the first run and processed with the combination of extracted ion chromatograms and LC-UV chromatograms. These processes were able to find accurate molecular masses of possible degradation products. Then, the accurate MS/MS data acquired through data-dependent analysis mode in another run facilitated the structural elucidations of degradation products. As a result, a total of 11 degradation products of danofloxacin were detected and characterized using the developed method. Overall, this analytical strategy enables the acquisition of accurate-mass LC/MS data, search of a variety of degradation products through the post-acquisition processes, and effective structural characterization based on elemental compositions of degradation product molecules and their product ions. The ability to measure degradation products via tandem mass spectrometry coupled with accurate mass measurement, all in only two experimental runs, is one of the most attractive features of this methodology. The results demonstrate that use of the LC/MS-IT-TOF approach appears to be rapid, efficient and reliable in structural characterization of drug degradation products.

Keywords Ion trap/time-of-flight mass spectrometry · Degradation product · Accurate mass measurements · Structural characterization · Danofloxacin

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Introduction

The identification of drug degradation products plays an important role in the drug discovery and development processes. Understanding the drug degradation profiles is also critical to the safety and potency assessment of drug. Thus, the International Conference on Harmonization guideline requires that stress testing be carried out to elucidate the stability characteristics of drug substances and medicinal products [1]. Information on the stability of the drug substances is an integral part of the systematic approach to stability evaluation. The degradation products

usually arise from the ingredients used in dosage formulation and/or in the process of formulation where temperature, humidity, and light may all play a part [2]. The degradation products are able to be generated from hydrolysis, oxidation, adduct formation, dimerization, rearrangement, and often the combination of these processes. To accelerate drug development, various stress-testing protocols had been designed to emulate stresses the compound may experience during manufacturing and storage conditions [1]. These methods expose drug to forced degradation conditions such as acid, base, oxidation, and exposure to light.

LC-MS/MS has become the technique of choice in the degradation product studies due to its selectivity, sensitivity, and speed of analysis. There have been a number of reports in the literature that applied LC-MS/MS for characterization of degradation products [3–6]. In 2000, Wu [7] reviewed the application of LC-MS/MS in the analysis of drug degradation products in pharmaceutical formulations under various stress conditions (oxidation, hydrolysis, dimerization, and adduct formation with excipients). However, the methodology of this instrument requires numerous LC runs to acquire mass on multiple fragment ions of degradation products. Currently, ion trap (IT) and time-of-flight (TOF) mass spectrometers are often used for the detection of degradation products [8–12]. IT can yield information regarding molecular structure, and it is highly efficient in the structural analysis of degradation products because of its MS^n capability [8]. Moreover, the current ion trap mass spectrometer is able to conduct automated data-dependent analyses [9]. The TOF mass spectrometer provides high-resolution analysis with mass accuracy, which increases the possibility of determination of the elemental composition of degradation products and provides high specificity in their detection [10–12]. However, IT cannot provide information with high resolution and cannot ensure good precursor ion selectivity.

One alternative solution is the use of hybrid instruments, such as the triple-quadrupole/time-of-flight (QqTOF) and LTQ-Orbitrap, which can provide structural information from fragmentation and accurate mass measurements for drug degradation products. Both instruments have been shown to enable fast, sensitive and accurate detection and identification of drug degradation products [13–16]. One limitation of the QqTOF mass spectrometry was the narrow ion abundance range over which accurate mass measurements could be made with a high degree of certainty [17, 18]. As one of the latest LC/MS instrumentation designs, hybrid ion trap/time-of-flight mass spectrometry coupled with high-performance liquid chromatography (LC/MS-IT-TOF) provides high sensitivity and accuracy. In particular, multiple scans of degradation products in MS and MS^2 modes and accurate mass measurements can be performed simultaneously through data-dependent acquisition. Although LC/MS-IT-TOF has the potential as one of the most effective techniques for the determination of unknown compounds, its application to drug degradation products characterization is much less routine than for the drug metabolites characterization [19–21].

In the present study, we explored an integrated approach for degradation products detection and structural characterization using the LC/MS-IT-TOF and post-acquisition data processing techniques. The effectiveness of this integrated approach was evaluated using danofloxacin as a model compound. Danofloxacin, 7-[(1*S*,4*S*)-5-methyl-2,5-diazabicyclo[2,2,1] hept-2-yl]-1-cyclopropyl-6-fluoro-1,4-dihydro-oxo-3-quinolinecarboxylic acid, is a fluoroquinolone antibacterial which has been developed specifically for veterinary use. Danofloxacin has been studied for use in cattle, pigs, chickens and sheep for the control of respiratory and enteric bacterial infection [22–24]. It is highly effective against many Gram-positive and -negative pathogens as well as mycoplasmas. Among side effects induced by fluoroquinolones, phototoxic reactions are by far the most common. Fluoroquinolones can undergo a variety of photochemical processes such as generation of singlet oxygen, production of superoxide, decarboxylation, defluorination, and oxidation of an amino subsistent at C7 [25]. Some photoproducts of fluoroquinolones have also been characterized by mass spectrometry [26, 27]. However, there is no report concerning the stability of danofloxacin.

Experimental

Chemicals

Danofloxacin (99.8%) was obtained from China Institute of Veterinary Drug Control (Beijing, China). The stock solution of danofloxacin was prepared by dissolving the compound in water at the concentration of 1.0 mg/ml. HPLC-grade acetonitrile was purchased from Fisher Chemicals Co. (NJ, USA). Water was freshly prepared with the Millipore water purification system (MA, USA). All other chemicals and reagents were of the highest analytical grade available.

Stress studies

The stress studies were carried out under the conditions of hydrolysis, oxidation, and photolysis. For all of the solution stability studies, the samples were prepared by mixing a dilution solution of danofloxacin (200 mg/l) with various media (1:1; *v*/*v*). Acidic and alkaline hydrolysis were carried out in 0.1 N HCl and 0.1 N NaOH, respectively, whereas neutral hydrolysis was performed in water. All the

hydrolytic studies were conducted at 80 °C for 24 h. The oxidative study was carried out in 30% H_2O_2 at room temperature for 12 h. The photostability testing was carried out by exposing the solution of drug in water to UV fluorescent light (208 Wh/m^2) at room temperature for 12 h. All the stressed samples were withdrawn at suitable time intervals and diluted ten times with water before injection into the LC/MS-IT-TOF.

Analytical system

For the characterization of danofloxacin and its degradation products, hybrid IT/TOF mass spectrometry coupled with a high-performance liquid chromatography system was used (Shimadzu Corp., Kyoto, Japan). The liquid chromatography system (Shimadzu) was equipped with a solvent delivery pump (LC-20AD), an autosampler (SIL-20AC), a DGU-$20A_3$ degasser, a photodiode array detector (SPD-M20A), a communication base module (CBM-20A) and a column oven (CTO-20AC). The separation was performed on a ODS-C18 column (150 mm×2.0 mm I.D.; particle size 5 μm) using a gradient elution consisting of mobile phase A (0.1% formic acid) and mobile phase B (acetonitrile). The gradient was as follows: 0–10 min, an isocratic elution of 10% B; 10–15 min, a linear gradient to 30% B; 15–20 min, an isocratic elution of 30% B; 20–20.1 min, a gradient back to 10% B. The whole analysis took 25 min. The injection volume was 20 μl, the flow rate was 0.2 ml/min, and the photodiode array detector detection was performed from 200 to 400 nm. The sample chamber in the autosampler was maintained at 4 °C, while the column was set at 40 °C.

The total effluent from the detector was transferred directly to the hybrid IT/TOF mass spectrometer without splitting. The mass spectrometer was equipped with an electrospray ionization source and operated in the positive mode. Mass spectrometric analyses were carried out on full-scan MS with a mass range of *m/z* 100–500 and data-dependent MS/MS acquisition on the suspected precursor ions. Nitrogen was used as the nebulizing gas set at 1.5 L/min. The capillary and skimmer voltages were set at 4.5 KV and 1.6 KV, respectively. The curved desolvation line (CDL) and heat block temperatures were both maintained at 200 °C. The MS^2 spectra were produced using collision-induced dissociation (CID) of the selected precursor ions using argon as collision gas with relative energy of 50%. The ion accumulation time were set at 50 ms, the precursor ion isolation width at 2 Da. External mass calibration was carried out prior to data acquisition using direct infusion of a reference standard from *m/z* 50 to 1,000. The reference standard was sodium trifluoroacetic which consisted of 0.25 mL/L trifluoroacetic acid and 0.1 g/L sodium hydrate. The flow rate of the infusion pump was 5 μL/min. All calculated mass error were less than 5 ppm after mass calibration with the reference standard.

Data acquisition and processing were carried out using the LC/MS solution version 3.41 software supplied with the instrument. Any mass numbers corresponding to particular elemental compositions were also calculated by the formula predictor, and would generate more than one formula proposed by the software. Therefore, accuracy error threshold of ±10 ppm was set as a limit to the calculation of possible elemental compositions. The other following conditions for calculating elemental compositions were taken into consideration: the upper limits on the number of C, H, O, N, F atoms, C/H ratios, nitrogen rule and range of double-bond equivalent.

Analytical strategy

The first step of this strategy was to obtain the accurate full-scan MS data using a simple and generic acquisition method. The first run analysis provided chromatographic behavior and accurate mass measurements for all existed ions. To search carefully possible accurate molecular masses of degradation products, post-acquisition data mining using the combination of accurate extracted ion chromatograms (EIC) and LC-UV chromatograms were performed. The retention times of potential degradation products were determined from the EIC and LC-UV chromatogram comparisons between the standard solution and the drug treated samples. Then, the accurate measured mass of each potential degradation products was ascertained by the MS spectrum. If a peak appeared from the LC-UV chromatograms and the accurately measured mass in the agreed with the theoretical mass to within 10 ppm based on the predicted formula, then the peak was proposed as the possible degradation product. If the peak was absent from the LC-UV chromatograms, but a peak appeared from the accurate EIC and the accurately measured mass in the MS spectrum agreed with the theoretical mass to within 10 ppm, then the peak was also proposed as the possible degradation product.

Once a degradation product ion was found by these data processing techniques, its product ion spectrum could be acquired in an addition MS/MS scanning analysis through a data-dependent analysis mode. Structures of the detected degradation products were characterized on the basis of the mass shift from the drug, molecular formulae derived from the accurate mass measurements, and the interpretation of accurate MS/MS spectra. The integrated strategy applied to the detection and structural characterization of degradation products using the LC/MS-IT-TOF and data mining techniques is illustrated in Fig. 1.

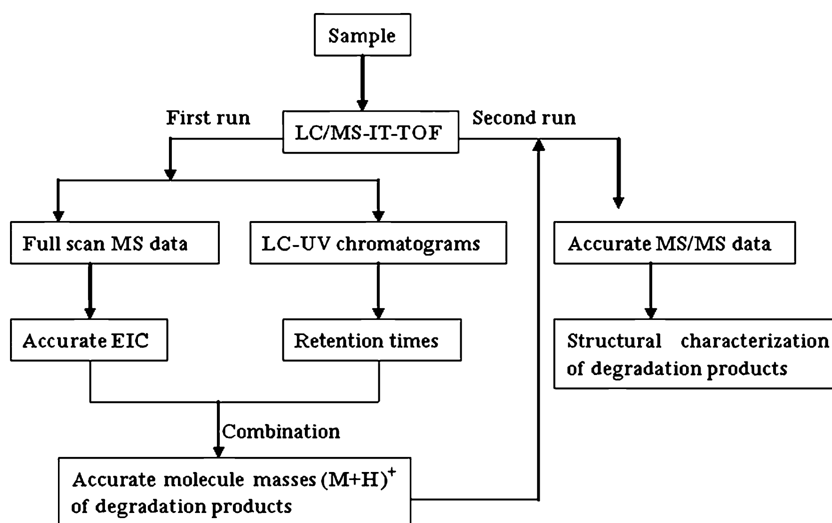


Fig. 1 An integrated method for degradation product detection and characterization using hybrid ion trap/time-of-flight mass spectrometry coupled with high-performance liquid chromatography (LC/MS-IT-TOF) and post-acquisition data mining techniques

Results and discussion

Fragmentation of danofloxacin

An initial step in elucidating structures of degradation products of danofloxacin is to understand the fragmentation pattern of the drug substance. The detailed mass spectrometry analysis of the fragmentation pattern of danofloxacin provides a basis for assessing structural assignment for the degradation products. The accurate MS, MS^2 spectra, and proposed fragmentation pathways of danofloxacin are shown in Fig. 2. These spectra were also used as references to examine the high resolution

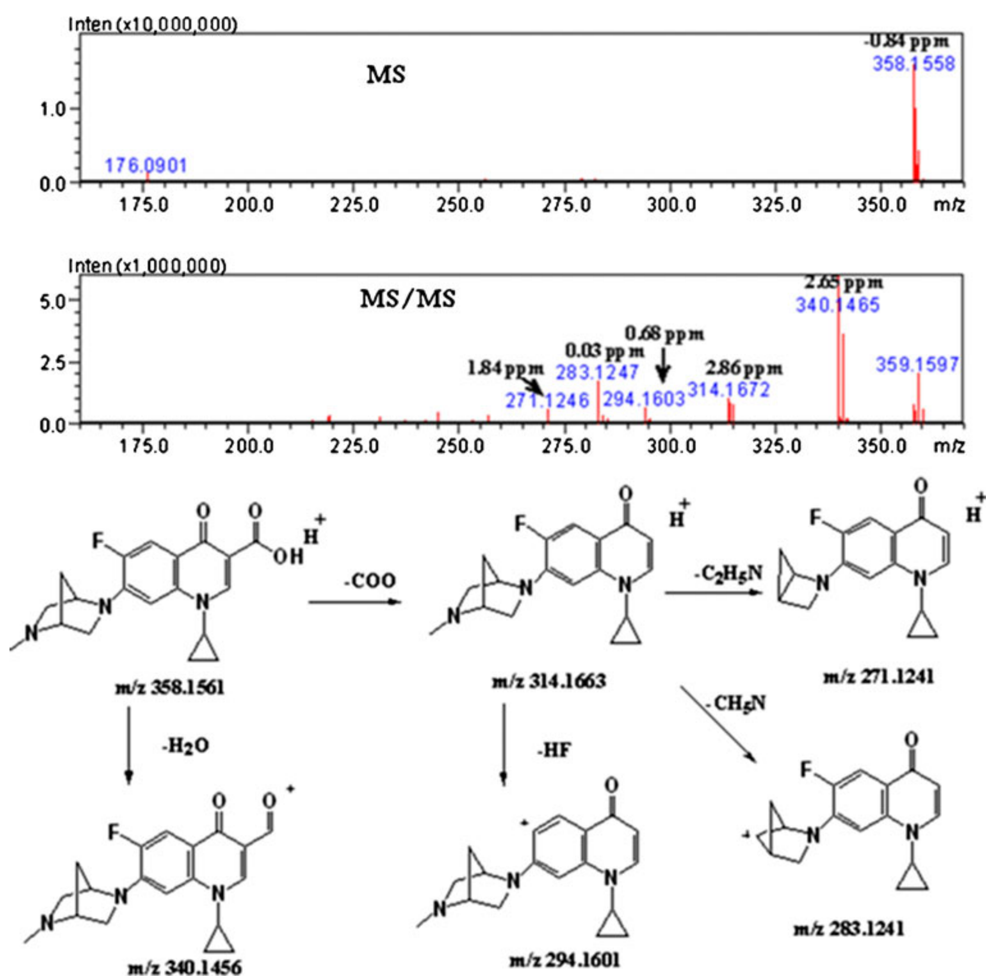


Fig. 2 Accurate MS, MS^2 spectra and proposed fragmentation pathways of danofloxacin

and mass accuracy of the instrument. Figure 2 shows the errors between measured and predicted values of protonated danofloxacin and product ions ranged from −0.84 to 2.86 ppm, indicated relatively good accuracy. For all product ions and their error ranges set less than 10 ppm, there are few different compositions that satisfy the requirement of elemental composition, so the structure of product ions can be obtained with a high degree of confidence base on the structure of parent drug. As shown in Fig. 2, protonated danofloxacin lost COO to form *m/z* 314, and then lost hydrofluoric acid to form *m/z* 294. The loss of H_2O from the carboxylic acid formed the predominant peak at *m/z* 340. The loss of C_2H_5N and CH_5N from the *m/z* 314 formed the product ions at *m/z* 271 and 283, respectively. All the proposed structures of the product ions were consistent with the results from the accurate mass measurements.

Elemental compositions of degradation products

Because the drug may produce some unexpected or uncommon degradation products, baseline chromatographic separation of these peaks was a necessary prerequisite for rapid identification of these degradation products. A 25-min acetonitrile gradient provided a suitable resolution in this work. From the chromatography, the optical spectrum of each peak provided retention time and the maximal absorption wavelength. The forced degradation samples of danofloxacin (neutral, acidic, basic, oxidative and photolytic) were analyzed by LC/MS-IT-TOF to detect the degradation products. The representative HPLC chromatograms of danofloxacin at each stress conditions are shown in Fig. 3. After a 24-h exposure to the hydrolytic medias (neutral, acidic, basic) at 80 °C, no any degradation

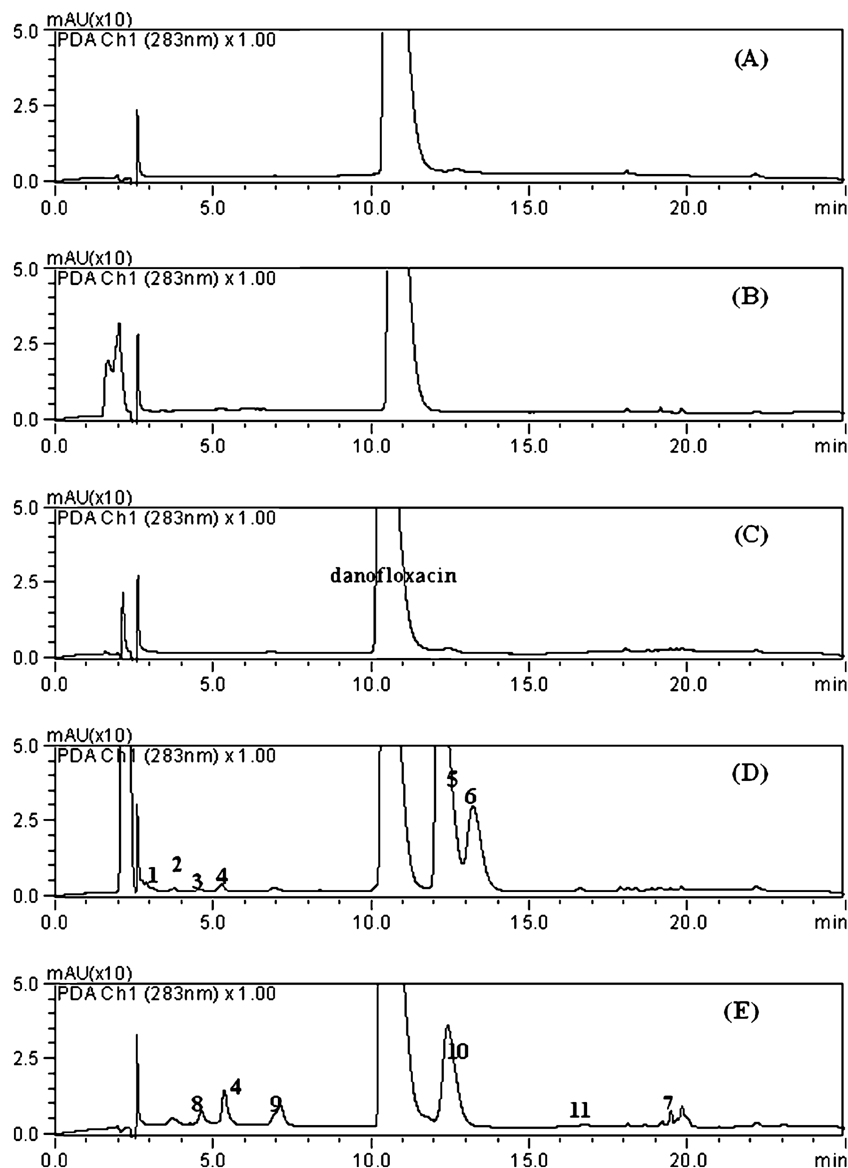


Fig. 3 LC-UV chromatograms of the stressed danofloxacin by H_2O for 24 h (**a**); 0.1 N HCl for 24 h (**b**); 0.1 N NaOH for 24 h (**c**); 30% H_2O_2 for 12 h (**d**); as well as UV fluorescent light at room temperature for 12 h (**e**)

products of danofloxacin was observed according the magnified UV absorption scale. The results indicated that danofloxacin was stable to thermal, acidic and basic stress. However, the drug showed degradation under oxidative and photolytic conditions. Using the analytical strategy, seven and six degradation products under the oxidative and photolytic conditions were detected rapidly in the first run by LC/MS-IT-TOF, respectively. The accurate extracted mass chromatograms of the degradation products of danofloxacin under the oxidative and photolytic conditions are shown in Fig. 4. The predicted elemental compositions, measured accurate masses and theoretical masses, and the mass errors of the degradation products are indicated in Table 1. The theoretical and measured masses agree to within less than 10 ppm, providing support for the proposed elemental compositions of the degradation products.

Structural elucidation of degradation products

To further elucidate the structure of these degradation products, the accurate MS^2 spectra of the degradation products were acquired in an additional run by LC/MS-IT-TOF. This enabled to determine the elemental compositions for the product ions of degradation products. The accurate MS^2 spectra and corresponding to mass errors between measured and theoretical masses of degradation products of danofloxacin are shown in Fig. 5.

Compound 1

Compound 1 had an retention time of 2.6 min and showed $[M+H]^+$ ion at m/z 390, which was 32 Da higher than that of danofloxacin, suggesting that it was a di-oxidation product of danofloxacin. The product ion at *m*/*z* 346.1338 had an elemental composition of $C_{18}H_{19}N_2O_4F$ (4.33 ppm; see Fig. 5), likely to have resulted from the loss of CH_2NO from the Compound 1 on the basis of elemental compost ion measurement of *m*/*z* 390.1454, indicating that an oxygen atom was located on side chain of diazabicyclo. The product might result from the oxidation of cleavage of the diazabicyclo according to a mechanism proposed by Mella et al. [28]. The loss of H_2O from the fragment ion at

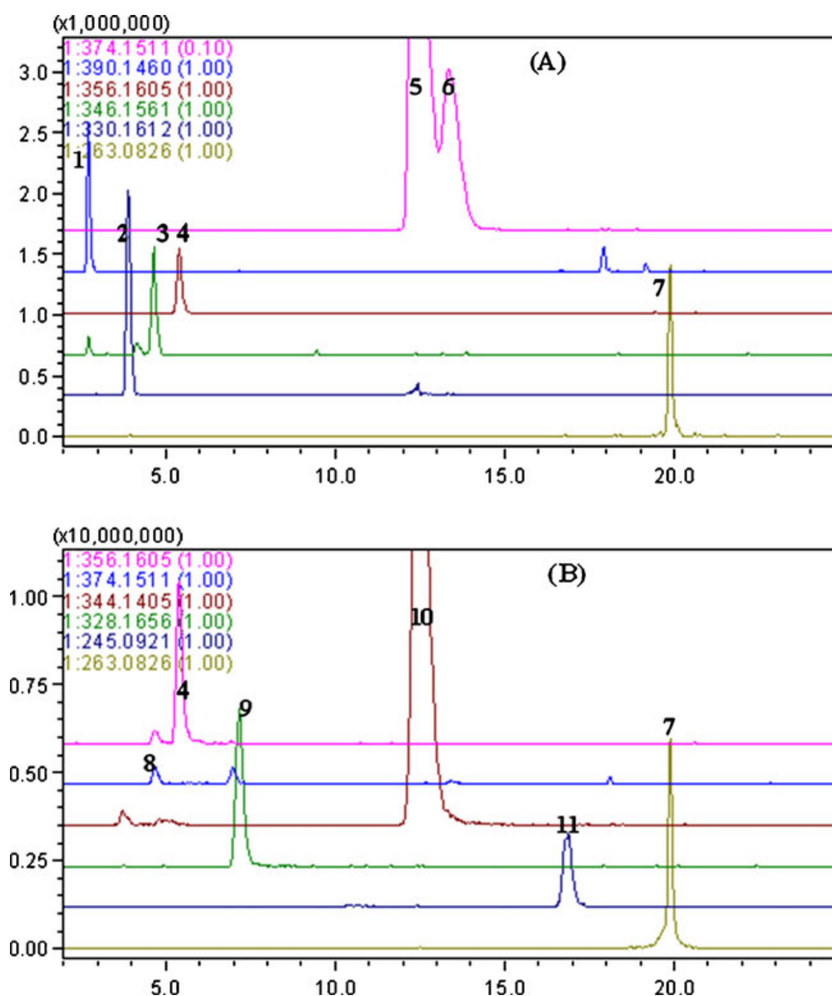


Fig. 4 The accurate extracted mass chromatograms (EIC) of degradation products of danofloxacin in 30% H_2O_2 for 12 h (**a**) and UV fluorescent light at room temperature for 12 h (**b**)

Table 1 The retention times (RT), measured accurate masses, predicated elemental compositions, theoretical masses, double bond equivalents (DBE) and mass errors of degradation products of danofloxacin

Degradation products	RT (min)	Measured mass (Da)	Elemental compositions ($[M+H]^+$)	DBE	Theoretical mass (Da)	Error (mDa)	Error (ppm)
1	2.6	390.1454	$C_{19}H_{21}N_3O_5F^+$	11	390.1460	–0.6	–1.54
2	3.8	330.1601	$C_{18}H_{21}N_3O_2F^+$	10	330.1612	–1.1	–3.33
3	4.7	346.1541	$C_{18}H_{21}N_3O_3F^+$	10	346.1561	–2.0	–5.78
4	5.3	356.1598	$C_{19}H_{22}N_3O_4{}^+$	11	356.1605	–0.7	–1.97
5	12.5	374.1501	$C_{19}H_{21}N_3O_4F^+$	11	374.1511	–1.0	–2.67
6	13.2	374.1496	$C_{19}H_{21}N_3O_4F^+$	11	374.1511	–1.5	–4.01
7	19.7	263.0831	$C_{13}H_{12}N_2O_3F^+$	9	263.0826	0.5	1.90
8	4.2	374.1521	$C_{19}H_{21}N_3O_4F^+$	11	374.1511	1.0	2.67
9	7.1	328.1676	$C_{18}H_{22}N_3O_3{}^+$	10	328.1656	2.0	6.09
10	12.3	344.1411	$C_{18}H_{19}N_3O_3F^+$	11	344.1405	0.6	1.74
11	16.7	245.0934	$C_{13}H_{13}N_2O_3{}^+$	9	245.0921	1.3	5.30

m/z 346 formed the predominant peak at *m/z* 328. However, the position of the other oxygen atom could not be proposed due to the lack of other product ions in the MS/MS spectrum of Compound 1.

Compound 2

Compound 2 was eluted at an retention time of 3.8 min and had a measured elemental composition of $C_{18}H_{21}N_3O_2F$ ($[M+H]^+$ ion at *m/z* 330). The MS^2 spectrum of Compound 2 showed three major product ions at *m/z* 310, 299, and 287 were all 16 Da higher than product ions at *m/z* 294, 283, and 271 of danofloxacin, respectively, indicating that an oxygen atom had not occurred on the diazabicyclo moiety. Therefore, Compound 2 was an oxidation products followed decarboxylation of danofloxacin. The elemental compositions of the proudct ions at *m/z* 235.0868 and 194.0472 were $C_{12}H_{12}N_2O_2F$ (predicted 235.0877 Da) and $C_9H_7N_2O_2F$ (predicted 194.0486 Da), respectively, according to the formula predictor software, indicating that cyclopropyl was lost from *m/z* 235 to form *m/z* 194. The presence of the *m/z* 194 ion corresponded to $C_9H_7N_2O_2F$ (predicted 194.0486 Da) indicated that oxygen atom had occurred on the quinoxaline ring. However, the exact position of the oxygen atom on the ring could not be determined by mass spectrometry.

Compound 3

Compound 3 was eluted at an retention time of 4.7 min and had a measured elemental composition of $C_{18}H_{21}N_3O_3F$ ($[M+H]^+$ ion at m/z 346). It fragmented readily to give an abundant product ion at *m/z* 329 corresponding to the loss of OH radical from the precursor ion, suggesting an oxygen atom of Compound 3 was located on the nitrogen at position 5 of diazabicyclo. The loss of OH radical is a fragmentation reaction for N-oxides [29]. The loss of C_2H_6N and C_5H_7N from the ion *m/z* 329 leads to product ions at *m/z* 285 and 248, respectively. The elemental compositions of the product ions at m/z 248.0935 and 192.0696 were $C_{13}H_{13}N_2O_2F$ (predicted 248.0956 Da) and $C_{10}H_9N_2OF$ (predicted 192.0696 Da), respectively, according to the formula predictor software, indicating that C_3H_4O was lost from *m/z* 248 to form *m/z* 192. This indicates another oxygen atom had occurred on the cyclopropyl. There are other low intensity fragment ions in the MS^2 of Compound 3. For example, the ion *m/z* 329 generated the product ion at *m/z* 309 when it lost one fluorine atom. Therefore, the Compound 3 was identified as a di-oxidation products followed decarboxylation of danofloxacin.

Compound 4

Compound 4 was eluted at an retention time of 5.3 min and had a measured elemental composition of $C_{19}H_{22}N_3O_4$ ($[M+H]^+$ ion at *m/z* 356). This compound corresponded to the addition of a hydroxyl followed by the loss of a fluorine atom on the danofloxacin. It fragmented readily to yield dominant ion at *m/z* 338, a loss of H_2O from the parent ion. Loss of COO from the precursor ion (*m/z* 356) leaded to a product ion at *m/z* 312 (see Fig. 5.). Further loss of CH_5N and C_2H_5N from the ion resulted ions at *m/z* 281 and 269, respectively. The breaking of the carbon-nitrogen of the diazabicyclo of the ion at *m/z* 338 generated the fragment ion at *m/z* 243. The elemental compositions of the fragment ions at *m/z* 243.0758 and 202.0366 were $C_{13}H_{11}N_2O_3$ (predicted 243.0764 Da) and $C_{10}H_6N_2O_3$ (predicted 202.0373 Da), respectively, according to the formula predictor software, indicating that cyclopropyl was lost from *m/z* 243 to form *m/z* 202. Moreover, the

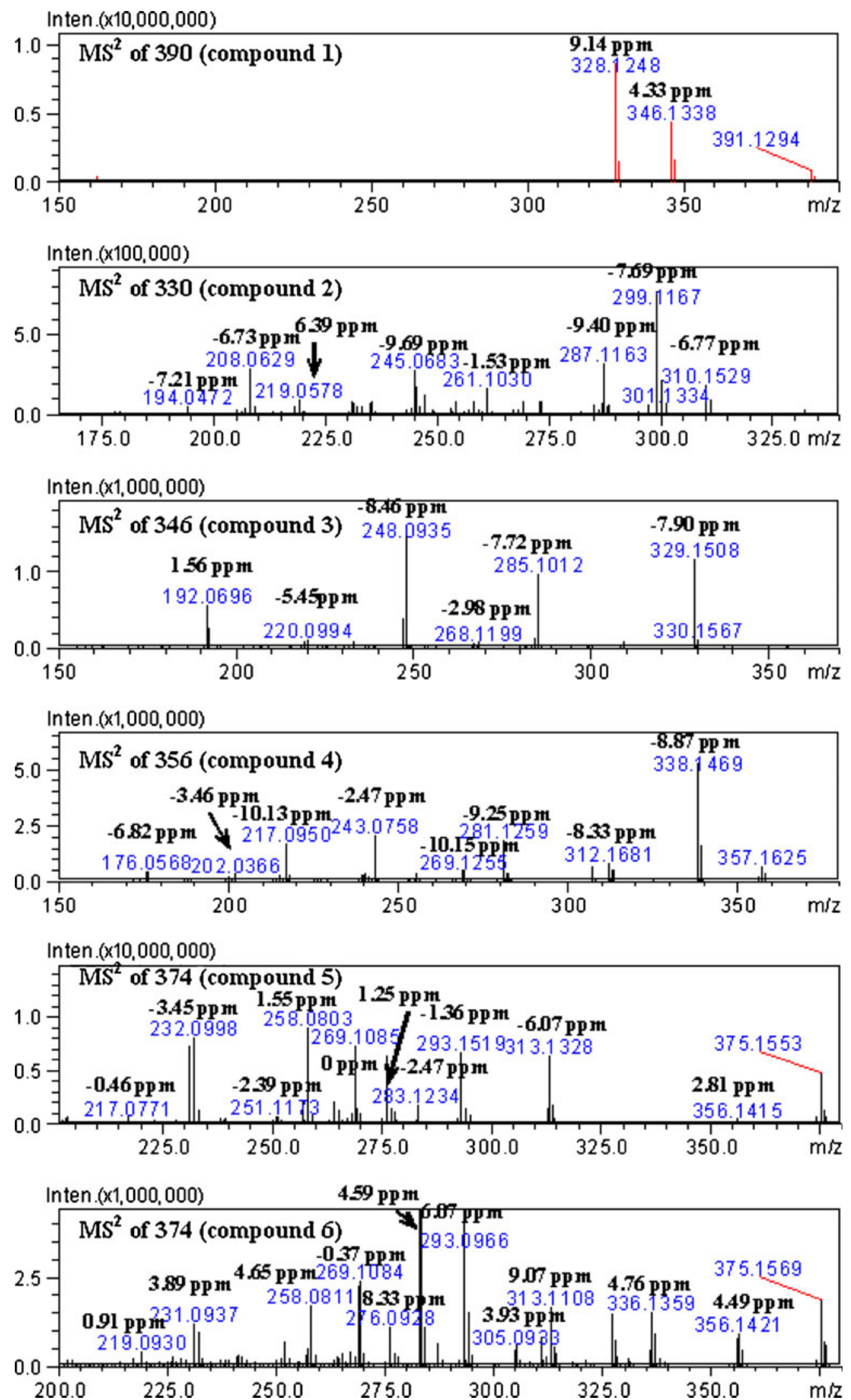
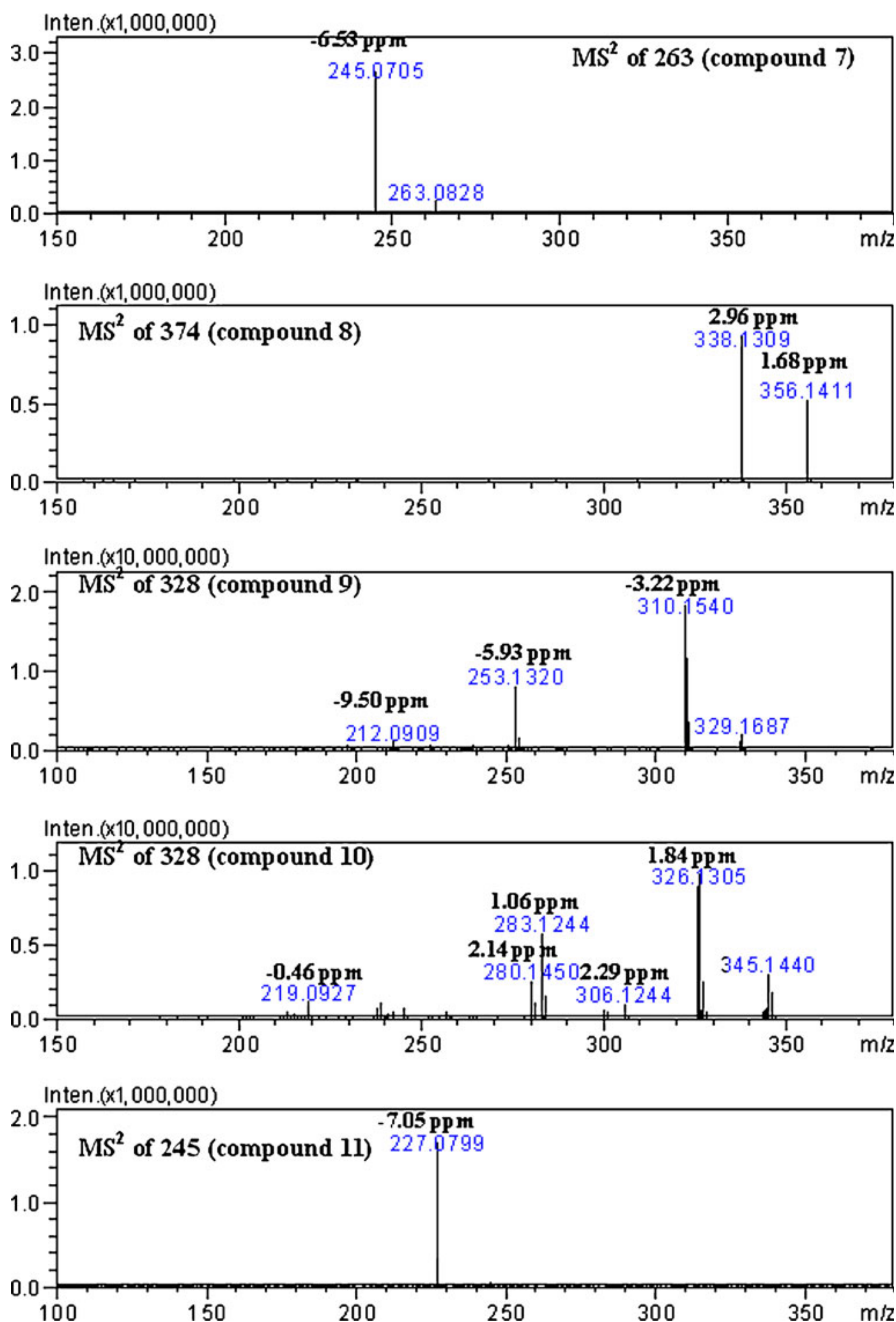


Fig. 5 Accurate MS^2 spectra and corresponding to mass errors between measured and theoretical masses of degradation products of danofloxacin

Fig. 5 (continued)



presence of the *m*/*z* 176 ion corresponded to $C_9H_8N_2O_2$ (predicted 176.0553 Da) indicated that oxygen atom had occurred on the quinoxaline ring. Taking into the mechanism proposed by Mella et al. [28], the product was identified as position of hydroxyl followed by the loss of a fluorine atom at position 6 on the quinoxaline ring.

Compounds 5 and 6

Compounds 5 and 6 were eluted at the retention times of 12.5 and 13.2 min, respectively. Both compounds showed similar protonated molecular ion at *m*/*z* 374.1501 and *m*/*z* 374.1496 and the same predicated elemental composition of

$C_{19}H_{21}N_3O_4F$, 16 Da higher than that of danofloxacin, suggested that they were the oxidized products via the addition of an oxygen atom. The mass fragmentation pattern for Compound 5 is almost same from that for Compound 6. Both compounds yielded the same product ions of *m*/*z* 356, 283, 276, 258, 232, and 231. Compounds 5 and 6 formed a common product ion of *m*/*z* 283, which was also observed in the product ion of danofloxacin. This indicated this part of danofloxacin was intact, and the oxidation could occur only on the diazabicyclo moiety. Although Compounds 5 and 6 yielded the same mass numbers for *m*/*z* 313 and 293, the accurate mass measurements of the fragment ions of *m*/*z* 313 and 293 were different (see Fig. 5). For Compound 5, the elemental compositions of the product ions at *m*/*z* 313.1328 and 293.1519 were $C_{18}H_{18}N_2O_2F$ (predicted 313.1347 Da) and $C_{18}H_{19}N_3O$ (predicted 293.1533 Da), respectively. However, for Compound 6, the elemental compositions of the fragment ions at *m*/*z* 313.1108 and 293.0966 were $C_{17}H_{14}N_2O_3F$ (predicted 313.1087 Da) and $C_{17}H_{13}N_2O_3$ (predicted 293.0941 Da), respectively. In addition, the loss of CHNO from the ion *m*/*z* 356 of Compound 5 leads to product ion at *m*/*z* 313, which was same as the fragmentation pattern of Compound 1. Therefore, the Compound 5 was identified as oxidation of cleavage of the diazabicyclo of danofloxacin. For Compound 6, the *m*/*z* 327 fragment was due to the loss of CH_5NO which indicated the oxygen atom on the nitrogen at position 5 of diazabicyclo. Therefore, this product was identified as oxidation of the nitrogen into N-oxide.

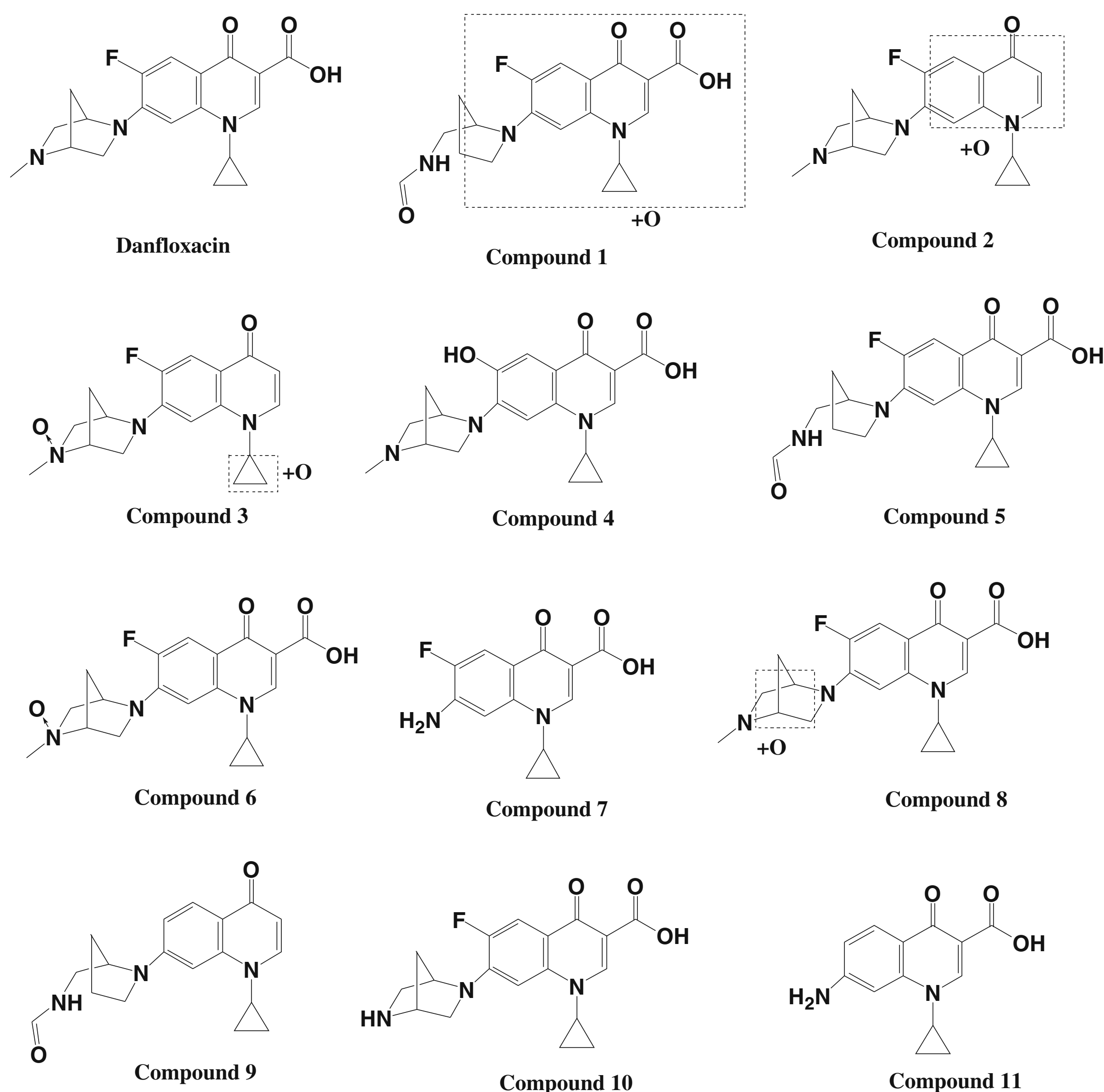


Fig. 6 Structural assignments of degradation products of danofloxacin

Compound 7

Compound 7 was eluted at an retention time of 19.7 min and had a measured elemental composition of $C_{13}H_{12}N_2O_3F$ ($[M+H]^+$ ion at *m/z* 263). This indicated that Compound 7 was the breaking of diazabicyclo ring of danofloxacin. This pathway was reported in the literature for many fluoroquinolones such as ciprofloxacin [26, 28]. It fragmented readily to give a dominant ion at *m/z* 338, a loss of H_2O from the parent ion. Therefore, Compound 7 was characterized as 7-amino-1-cyclopropyl-6-fluoro-1,4-dihydro-oxo-3-quinolinecarboxylic acid.

Compound 8

Compound 8 was eluted at an retention time of 4.2 min and had a measured elemental composition of $C_{19}H_{21}N_3O_4F$ ($[M+H]^+$ ion at *m/z* 374), which 16 Da higher than that of danofloxacin, suggested that it was the oxidized products via the addition of an oxygen atom. The product ion at *m/z* 356 was formed by the loss of H_2O from Compound 8; it lost a further H_2O to form *m/z* 338. Thus, we proposed oxygen atom would occur on the carbon of diazabicyclo moiety.

Compound 9

Compound 9 was eluted at an retention time of 7.1 min had a measured elemental composition of $C_{18}H_{22}N_3O_3$ ($[M+H]^+$ ion at *m/z* 328). The product ion at *m/z* 310 was formed by the loss of H_2O from Compound 9; it lost a further C_2H_3NO to form *m/z* 253, indicating an oxygen atom of Compound 9 was located on side chain of diazabicyclo moiety. The product might result from the oxidation of cleavage of the diazabicyclo according to a mechanism proposed by Mella et al. [28]. The elemental compositions of the product ions at *m/z* 253.1320 and 212.0909 were $C_{16}H_{17}N_2O$ (predicted 253.1335 Da) and $C_{13}H_{12}N_2O$ (predicted 202.0944 Da), respectively, according to the formula predictor software, indicating that cyclopropyl was lost from *m/z* 253 to form *m/z* 212. Therefore, Compound 9 was identified as oxidation products followed by the loss of a fluorine atom and carboxylic acid of danofloxacin.

Compound 10

Compound 10 was eluted at a retention time of 12.3 min, had a measured elemental composition of $C_{18}H_{19}N_3O_3F$ ($[M+H]^+$ ion at *m/z* 374), which is 14 Da lower than that of danofloxacin, suggested that it was a demethyl product of danofloxacin. The MS^2 spectrum of Compound 10 showed product ions at *m/z* 326, 280 were also 14 Da lower than fragment ions at *m/z* 340, 294 of danofloxacin, respectively. Therefore, Compound 10 was identified as *N*-demethyldanofloxacin.

Compound 11

Compound 11 was eluted at an retention time of 16.7 min and had a measured elemental composition of $C_{13}H_{13}N_2O_3$ ($[M+H]^+$ ion at *m/z* 245). This indicated that Compound 11 was the breaking of diazabicyclo ring of danofloxacin. It fragments readily to give a dominant ion at m/z 338, a loss of H_2O from the parent ion. Therefore, Compound 11 was identified as 7-amino-1-cyclopropyl-1,4-dihydro-oxo-3-quinolinecarboxylic acid.

On the basis of accurate MS^2 spectra and elemental compositions of the degradation products, all eleven degradation products have been identified. Structural assignments of degradation products of danofloxacin under different forced conditions are proposed in Fig. 6.

Conclusion

In the present study, we explored a new analytical strategy for the rapid diction and structural characterization of degradation products under stressed conditions using hybrid ion trap/time-of-flight mass spectrometry and post-acquisition data mining techniques. The results have demonstrated several significant advantages of this approach. First, accurate full scan MS and MS/MS data of degradation products are acquired by only two experimental runs. Second, the detection of metabolites is accomplished primarily through the post-acquisition data processing with the combination of accurate EIC and LC-UV chromatograms. These data mining techniques are complementary and able to target both common and uncommon degradation products. Finally, the accurate MS/MS data acquired in the data-dependent analysis mode facilitate the fragmentation interpretation for structural elucidation of degradation product. The approach in structural characterization of drug degradation product using LC/MS-IT-TOF is a rapid, efficient and reliable analytical method, which may be useful in the drug discovery and development processes to screen the degradation pathways of drug.

References

1. ICH International Conference on Harmonisation of Technical requirements for registration of pharmaceuticals for human use, Stability testing of new drug substance and products (2000), 1–17
2. Erni F (1982) J Chromatogr 251:141–151
3. Feng W, Liu H, Chen G, Malchow R, Bennett F, Lin E, Pramanik B, Chan TM (2001) J Pharm Biomed Anal 25:545–557

4. Lee MS, Kerns EH (1999) Mass Spectrom Rev 18:187–279
5. Volk KJ, Hill ES, Kerns EH, Lee MS (1997) J Chromatogr B: Biomed Sci Appl 696:99–115
6. Zhang LK, Pramanik BN (2010) J Mass Spectrom 45:146–156
7. Wu Y (2000) Biomed Chromatogr 14:384–396
8. Piccinelli AL, Campone L, Piaz F, Cuesta-Rubio O, Rastrelli L (2009) J Am Soc Mass Spectrom 20:1688–1698
9. Chen T, Fu F, Chen Z, Li D, Zhang L, Chen G (2009) J Chromatogr A 1216:3217–3222
10. Mehta S, Shan RP, Priyadarshi R, Singh S (2010) J Pharm Biomed Anal 52:345–354
11. Ferrer I, Mezcua M, Gomez MJ, Thurman EM, Aguera A, Hernando MD, Fernandez-Alba AR (2004) Rapid Commun Mass Spectrom 18:443–450
12. Nagele E, Moritz R (2005) J Am Soc Mass Spectrom 16:1670–1676
13. Maquille A, Salembier S, Herent MF, Habib Jiwan JL (2010) J Photochem Photobiol A: Chem 214:224–229
14. Watanabe T, Okabayashi M, Kurokawa D, Nishimoto Y, Ozawa T, Kawasaki H, Arakawa R (2010) J Mass Spectrom 45:799–805
15. Thevis M, Thomas A, Schanzer W (2008) Mass Spectrom Rev 27:35–50
16. Eichhorn P, Ferguson PL, Perez S, Aga DS (2005) Anal Chem 77:4176–4184
17. Bristow T, Constantine M, Harrison M, Cavoit F. Rapid Commun Mass Spectrom 22:1213–1222
18. Koefeler HC, Gross ML (2005) J Am Soc Mass Spectrom 16:406–408
19. Liu Z, Huang L, Dai M, Chen D, Wang Y, Tao Y, Yuan Z (2008) Rapid Commun Mass Spectrom 22:1009–1016
20. Liu Z, Huang L, Dai M, Chen D, Tao Y, Wang Y, Yuan Z (2009) Rapid Commun Mass Spectrom 23:2026–2034
21. Liu ZY, Huang LL, Chen DM, Dai MH, Tao YF, Wang YL, Yuan ZH (2010) Anal Bioanal Chem 396:1259–1271
22. Mckellar QA, Gibson I, McCormack RZ (1998) Biophar Drug Dispos 19:123–129
23. Sappal R, Chaudhary RK, Sandhu HS, Sidhu PK (2009) Vet Res Commun 33:659–667
24. Rowan TG, Sarasola P, Sunderland SJ, Giles CJ, Smith DG (2004) Vet Rec 154:585–589
25. Zoltan T, Vargas F, Lzzo C (2007) Anal Chem Insights 2:111–118
26. Vasconcelos TG, Henriques DM, Konig A, Martins AF, Kummerer K (2009) Chemosphere 76:487–493
27. Budai M, Grof P, Zimmer A, Papai K, Klebovich I, Ludanyi K (2008) J Photochem Photobiol A: Chem 198:268–273
28. Mella M, Fasani E, Albini A (2001) Helv Chim Acta 84:2508–2519
29. Miao XS, Maech RE, Metcalfe CD (2003) Int J Mass Spectrom 230:123–133