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Short communication

Application of a hybrid ion trap/time-of-flight mass spectrometer in metabolite characterization studies: Structural identification of the metabolism profile of antofloxacin in rats rapidly using MS^n information and accurate mass measurements

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ABSTRACT

We describe herein, a very effective way in the rapid identification of metabolites for antofloxacin based on a hybrid ion trap (IT)/time-of-flight (TOF) mass spectrometer technique. The purified samples were separated by a reversed-phase C_{18} column under a gradient elution, antofloxacin and its metabolites were detected by the on-line IT/TOF detector in scan mode. The identification of the metabolites and elucidation of their structure were performed by comparing the changes in molecular masses (ΔM), calculating compound-based component by Formula Predictor software, and defining sites of biotransformation based upon mass shifts of diagnostic fragment ions according to the accurate MS^n spectral information. In this case, we used such strategies for the identification of the metabolism for antofloxacin, and six metabolites of antofloxacin were found in rats for the first time.

1. Introduction

Metabolite identification plays an important role at various stages of drug discovery and development. In preclinical development for a drug candidate, an early understanding of the in vivo and in vitro metabolic fate is essential since metabolites can contribute to pharmacological action or possess toxicological consequences independent of the parent drug [1].

Antofloxacin, a newly developed active quinolone carboxylic acid derivate, exhibits effective antibacterial activity through inhibiting aerobic and anaerobic Gram-positive and Gram-negative bacteria [2]. We had previously developed and validated a quantitative method for the determination of antofloxicin in plasma [3]. The aim of the present study was to elucidate the metabolism of antofloxicin in rats.

In general, LC–MS^n technique is always used to analyze drug metabolites in biological materials [4–6]. The IT technology is characterized by MS^n capabilities with unmatched sensitivity and fast data acquisition. However, IT analyzer has limited resolution, low-ion trapping capability, and space-charging effects that result in measurements lacking accuracy [7–10]. In order to overcome these shortcomings, time-of-flight (TOF) analyzer is connected to the system. The new hybrid TOF/MS has become increasingly popular because of its good resolution (10,000–15,000 FWHM), accuracy (<5 ppm), high transmission efficiency, theoretically unlimited mass range, and non-scanning nature [11,12]. As a demonstration of the capability of this new technology, we examined the metabolic pathways of the antibacterial drug antofloxacin in rats.

2. Experimental

2.1. Chemicals and reagents

Antofloxacin (purity > 99%) and its tablets were obtained from Anhui Global Pharmaceutical Co. Ltd. (Anhui, China). Each tablet contains 100 mg antofloxacin.

HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Analytical grade formic acid was purchased from Sigma Chemicals (St. Louis, MO). Water was collected from a Milli-Q Ultrapure water system with the water outlet operating at 18.2 MΩ (Millipore, Bedford, USA). Other chemicals and solvents were all of analytical grade.

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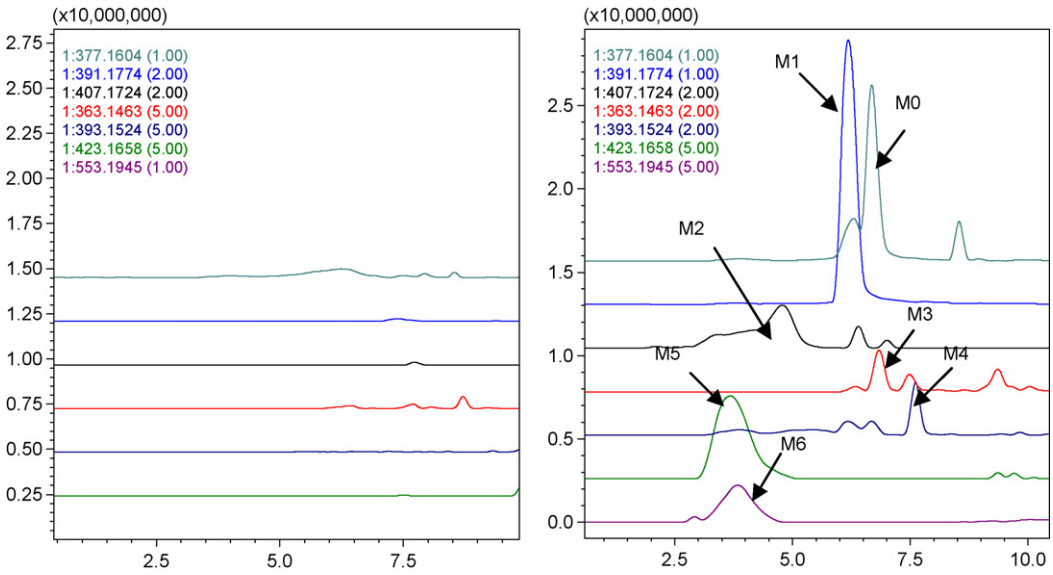

Fig. 1. Extracted ion chromatograms (EIC) of a blank bile sample and a dosed bile sample of rats.

2.2. Sample collection and preparation

Experimental animals: Animal studies were conducted according to protocols approved by the Review Committee of Animal Care and Use. Six male Sprague–Dawley rats (weighing 200–250 g) were fasted for 16–22 h with free access to water. A cannula was implanted under ether anesthesia for the entire duration of the surgery for the bile collection. The blank bile was collected before administration. After recovery from the surgery, the pre-prepared drug solution was administrated at a dose of 50 mg/kg. The bile was collected in Eppendorf 15 mL microcentrifuge polypropylene tube for 12 h, and the rats were given water occasionally during the course of bile collection.

In order to collect urine sample, another six rats were kept in individual metabolic cages, and orally administrated the same dose of antofloxacin as mentioned above. Urine samples were collected at 0–6, 6–12, 12–24, 24–48 h after dosing. All these samples were stored at −20 °C until analysis.

Purification of bile and urine samples: The bile and urine samples were centrifuged at 10,000 × *g* for 10 min. Solid-phase extraction with a C_{18} cartridge (Waters C_{18} Sep-Pak, 500 mg) was used to purify the above supernatants. Before extraction, the cartridge was conditioned with 1 mL of methanol followed by 1 mL of water. Then the sample was passed through the cartridge and washed with 2 mL of water to remove the impurity. One milliliter of methanol was added to elute the analytes. The methanol effluent was centrifugated at 40,000 × *g* for 10 min at 4 °C, the supernatants were stored at −20 °C until for LC-IT-TOF/MS analysis.

2.3. Instrumentation and conditions

HPLC experiments were conducted on a Shimadzu (Kyoto, Japan) HPLC system consisting of an LC-20AB binary pump, a DGU-14A degasser, a SIL-20AC autosampler and a CTO-20AC column oven. Chromatographic separation was achieved on a Shim-pack C_{18} column (150 mm × 4.6 mm ID, 5 μm, Shimadzu Co.) at 40 °C. The mobile phase (delivered at 0.2 mL/min) consisted of solvent A (H_2O, containing 0.05% HCOOH) and solvent B (CH_3OH). A binary gradient elution was performed: initial 15% B for 0.5 min, linear gradient 15–80% B from 0.5 to 10.0 min, isocratic 80% B from 10.0 to 11.0 min, then quickly returned to initial 15% B and maintained until 19 min for column balance. The effluent from the first 2 min was diverted to waste to minimize the contamination of the electrospray ionization (ESI) source.

MS^n analyses were conducted on a Shimadzu IT-TOF/MS (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source, and the optimized operating conditions were as follows: positive mode; electrospray voltage, 4.5 kV; nebulizer gas (N_2) flow, 1.5 L/min; nebulizer gas (N_2) flow, 5 L/min; trap cooling gas (Ar) flow, 95 mL/min; pressure of ion trap, 1.7×10^{-2} Pa; pressure of TOF region, 1.5×10^{-4} Pa; ion accumulated time, 30 ms; collision energy was set at 50% both for MS^2 and MS^3; scan range of *m/z* 250–700 for MS^1, 100–600 for MS^2, 50–500 for MS^3.

Metabolites identification was performed by MetID solution 1.0, and Shimadzu's Composition Formula Predictor software was used to provide chemical formula for antofloxacin and its metabolites.

3. Results and discussion

The urine and bile samples were subjected to LC-IT-TOF/MS using the method described in Section 2.3. And then, targeted data analysis was carried out with the aid of MetID solution software, which employs an extensive list of potential biotransformation reactions (e.g. methylation, hydroxylation, demethylation, etc.) to generate a series of extracted ion chromatograms (EIC) from post-acquisition data sets. Fig. 1 shows the EICs of a blank bile sample and a dosed bile sample of rat. Besides the parent drug, there were six new peaks which could be attributed to metabolites derived from the oxidation, reduction, combination of antofloxacin.

A prerequisite for the metabolite identification studies was to record the LC–MS^n spectrum of the parent compound under conditions of high mass resolution in order that the proposed pathways of fragmentation are fully supported by elemental composition data. Fig. 2 shows the product ion spectrum of antofloxacin resulting from one total scan comprised of MS^n mode. The fragment ion peaks at *m/z* 333.1712 and 276.1132 (loss of CO_2 and $C_5H_{12}N_2$, respectively) were abundant in the MS^2 spectrum, and the MS^3 spectra showed four diagnostic fragment ions of *m/z* 214.0608, 201.1024, 256.1064 and 171.0626. The molecular formulae and corresponding mass errors for these ions were predicted by Formula Predictor software (Fig. 2). Taken together, the fragmentation pathway could be predicted as what has been shown in Fig. 3.

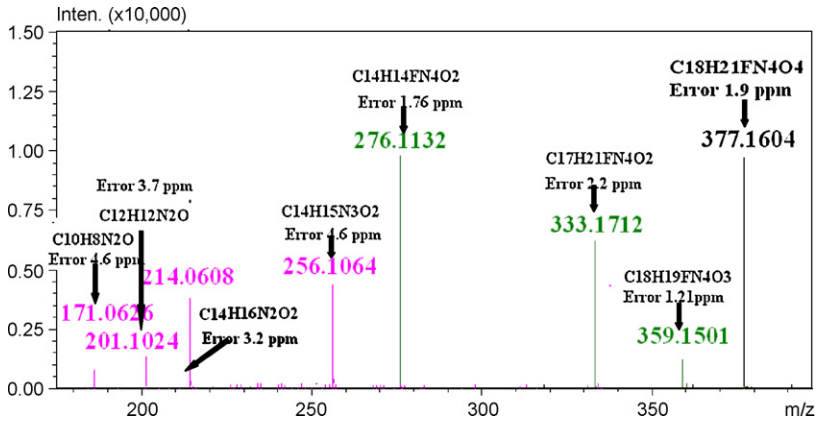

Fig. 2. Full scan accurate mass spectrum of antofloxacin (*m*/*z* 377.1604) acquired in the IT-TOF mass spectrometer using external mass calibration. The expanded mass range was shown for its product ions. Product ion assignment resulted from predictive fragmentation using Shimadzu's Composition Formula Predictor software.

M1, as a main metabolite appeared in bile and urine, was calculated as $C_{19}H_{23}FN_4O_4$ by the Formula Predictor software according to the accurate mass. Thus, M1 (addition of CH_2 to M0) was preliminary concluded as the methylating product of M0. In order to pinpoint the site of methylation in this metabolite, we compared the MS^n data from M1 with the corresponding data of the parent drug. The fragment obtained from the precursor (*m*/*z* 391.1774) showed a major product ion at *m*/*z* 359.1503, which could lead to six MS^3 product ions at *m*/*z* 331.1557, 302.0837, 283.0941, 214.0880, 201.1025 and 184.0888. On the basis of the elemental compositions of fragment ions and bond connectivities present in the parent molecule, the most likely methylating position was located at the carboxyl group. The possible structure and proposed fragmentation pathways of M1 are shown in Fig. 4.

The molecular ion of M2 (*m*/*z* 407.1724, $C_{19}H_{23}FN_4O_5$) was 15.9950 Da higher than that of the molecular ion of M1 and its main product ions at *m*/*z* 348.1236 and 293.0906. Calculating the formula by the Formula Predictor software, the product ions at *m*/*z* 348.1236 and 293.0906 were formed through neutral losing of C_2H_5NO and $C_5H_{10}N_2O$, respectively. In fact, the key fragment ion (*m*/*z* 293.0906) had permitted rapid localization of the site of hydroxylation on the N-methylpiperazine. The possible structure and proposed fragmentation pathways of M2 can be seen clearly from Fig. 4.

Likewise, metabolite M3 was calculated as $C_{17}H_{19}FN_4O_4$ (a net loss of CH_2 from M0) by the Formula Predictor software according to the accurate mass measurements, and was preliminary concluded as the demethylated product of antofloxicin. The spectra of MS^2 obtained from the precursor ion at *m*/*z* 363.1463 showed five major product ions at *m*/*z* 345.1330 (base peak), 299.1557, 276.1082, 262.1039 and 214.0610 (Fig. 4). We concluded that demethylating position should be located on the piperazine group through the product ion at *m*/*z* 276.1082, which also appeared in the MS^2 spectrum of antofloxacin.

The molecular ion of M4 (*m*/*z* 393.1524) could lead to four main MS^2 ions at *m*/*z* 376.1511, 356.1498, 328.1158 and 313.1042 and the fragment ion at *m*/*z* 376.1511 could lead to two MS^3 product ions at *m*/*z* 313.1042 and 269.1267. These results indicated that M4 was the hydroxylated product of M0, and the hydroxylated position was located on N-methylpiperazine group. The structure and the fragment pathway have been concluded and are shown in Fig. 4.

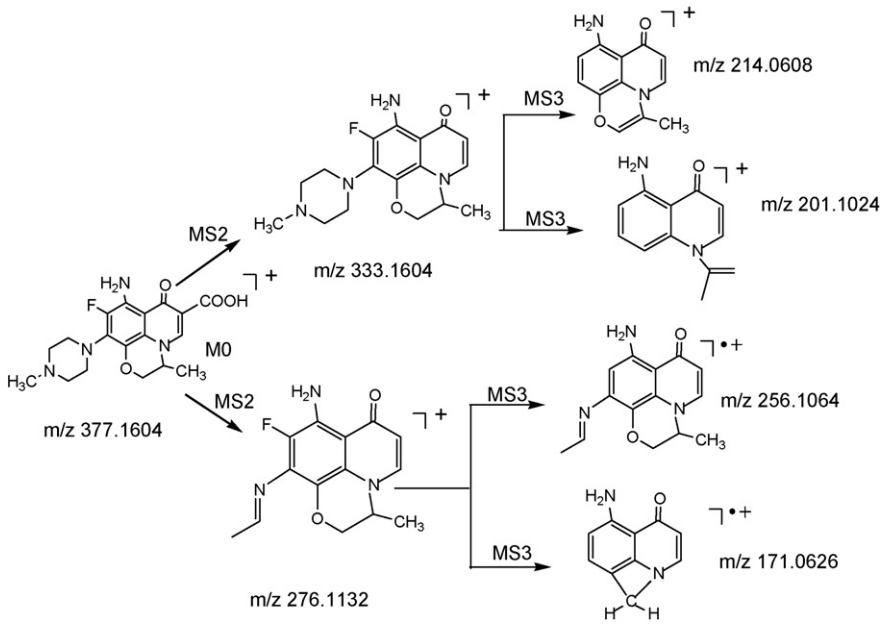

Fig. 3. Mainly proposed fragmentation pathways for antofloxacin resulting from collisional activation. The reaction mechanisms are predicted using Shimadzu's Composition Formula Predictor software.

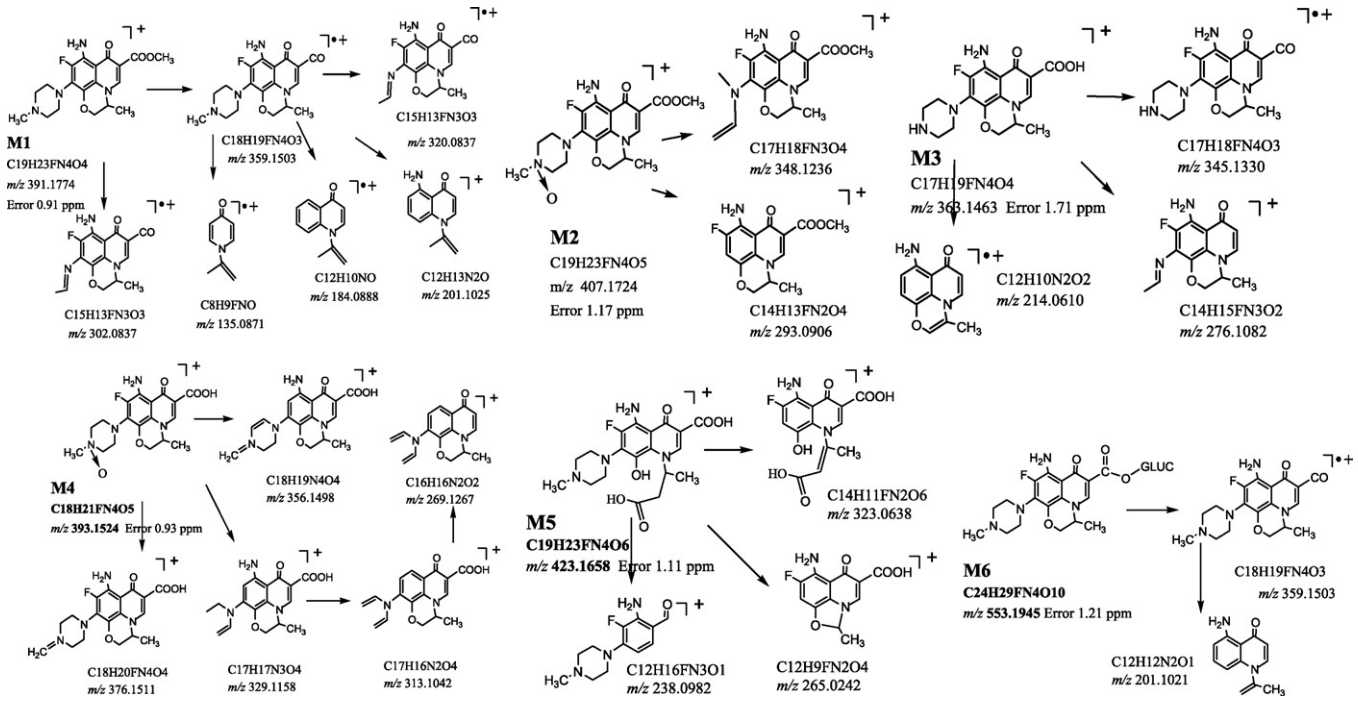

Fig. 4. The proposed fragmentation pathways and the structures of the six metabolites.

Mass spectra of M5 show a $[M+H]^+$ ion at *m*/*z* 423.1658 ($C_{19}H_{23}FN_4O_6$), which gave a molecular formula of suggesting the apposition of CH_2O_2. In the MS^2 experiment, major product ions at *m*/*z* 323.0683, 265.0243 and 238.0982 (base peak) were observed. The fragment ion at *m*/*z* 323.0683 should be formed via the loss of N-methylpiperazine by comparing the fragment ions derived from the parent, hence the reaction was not occurred on the piperazine group. According to the metabolism rule in vivo, we concluded that M5 may be formed by ring-opening and formic acid combination. In addition, the product ions *m*/*z* 265.0243 (loss of the N-methylpiperazine and acetic acid group) could also verify the structure and fragmentation pathway of M5 which are shown in Fig. 4. Similarly, the glucuronide conjugate of antofloxicin was observed as a major phase II metabolite with the $[M+H]^+$ ion at 553.1945. Generally speaking, the glucuronate always binds with amino or hydroxyl group according to the known common metabolic pathways. In order to pinpoint the site of glucuronic acid in this metabolite, the fragment information becomes very important. In this process, the characteristic fragment ion of the parent drug at *m*/*z* 359.1503 was also present in the MS^2 spectrum of M6. In addition, the MS^3 spectrum of this product ion (*m*/*z* 201.1021) was also identical to that of the same product ion from antofloxicin standard. All of these features suggested that the glucuronidation occurred at the carboxyl group.

In this study, we found that M1–M4 were appeared in rat urine and bile, but M5 and M6 only presented in rat bile. This study also indicated that the parent drug and all the metabolites could be detected until 12 h after administration. Besides, the metabolites in rat blood and feces were also studied, and the type of metabolites in the rat blood samples was similar to those in urine, and the metabolites detected in the rat feces samples were same as those in bile, whereas the amount of metabolites in the blood and feces were far less than the amount in urine and bile.

4. Conclusion

The metabolites of antibacterial drug antofloxacin in rat were studied for the first time by a new hybrid IT/TOF mass spectrometer. The MS^n data with high mass accuracy provided much formation to investigate the structures of metabolites under electrospray ionization. The experimental results indicate that the hybrid IT/TOF mass spectrometer is powerful and effective tool for in the detection and characterization of drug metabolites in samples of biological origin.

References

[1] A.S. Kalgutkar, J.R. Soglia, Exp. Opin. Drug Metab. Toxicol. 1 (2005) 91–142.
[2] X.Y. Pang, L. Liu, D.M. Zhang, G.J. Wang, L. Xie, X.D. Liu, J. Pharm. Pharmacol. 60 (2008) 1–4.
[3] J.H. Hu, L. Xie, X.D. Liu, J. Chin. Pharm. Univ. 37 (2006) 153–154.
[4] X.L. Wu, T.J. Hang, J.P. Shen, Y.D. Zhang, J. Pharm. Biomed. Anal. 41 (2006) 918–924.
[5] H. Chen, Y. Chen, P. Du, F. Han, J. Pharm. Biomed. Anal. 44 (2007) 773–778.
[6] G. Hoizey, A. Goglin, J.M. Malinovsky, A. Robinet, L. Binet, M.L. Kaltenbach, H. Millart, D. Lamiable, J. Pharm. Biomed. Anal. 42 (2006) 593–600.
[7] Y. Tohru, S. Hiroaki, T. Kazuo, J. Am. Soc. Mass Spectrom. 17 (2006) 67–74.
[8] W. Stephen, P.W. Holman, G. John Langley, Rapid Commun. Mass Spectrom. 22 (2008) 2355–2365.
[9] R. Philip, J. Tiller, L. Kerry, T. Fillgrove, Y. Sean, Rapid Commun. Mass Spectrom. 22 (2008) 1053–1061.
[10] B. Stahl, M. Steup, M. Karas, F. Hillenkamp, Anal. Chem. 63 (1991) 1463–1466.
[11] Z. Cui, J.A. Theruvathu, A. Farrel, A. Burdzy, L.C. Sowers, Anal. Biochem. 379 (2008) 196–207.
[12] G.C. Gil, Y.G. Kim, B.G. Kim, Anal. Biochem. 379 (2008) 45–59.