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Simultaneous Analysis of Moxifloxacin and Its Intermediate (4aS,7aS)-1H-octahydropyrrolo [3,4-b]pyridine by UHPLC Coupled with time-of-flight Mass Spectrometry

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***Abstract*—A fast UHPLC-MS method was developed to detect moxifloxacin and its intermediate (4aS,7aS)-1H-octahydropyrrolo [3,4-b]pyridine (intermediate 1) simultaneously for the first time. The moxifloxacin and intermediate 1 were separated within 2.0 min. The reliability of the method was demonstrated with the specificity, precision, and accuracy. Based on the peak area in the extracted ion chromatography (EIC), the linear regressions for moxifloxacin and intermediate 1 were both calibrated. In nine replicated trials, the relative standard deviation values for two analytes were both less than 5%. The average recovery of moxifloxacin at 30 ng/mL was 97.10%, while that of intermediate 1 at 3 ng/mL was 98.28%. The concrete samples demonstrated that this unique method can be applied in the producing process and quality control of moxifloxacin.**

***Index Terms*—Moxifloxacin, intermediate, (4aS,7aS)-1H-octahydropyrrolo[3,4-b]pyridine, UHPLC-MS.**

I. Introduction

Moxifloxacin is a quinolone/fluoroquinolone broad spectrum antibiotic that can be used to treat infections caused by Aerobic Gram-positive microorganisms including Corynebacterium species, Micrococcus luteus, etc., Aerobic Gram-negative microorganisms (Haemophilus influenzae, Haemophilus parainfluenzae, etc.) and other microorganisms such as Chlamydia trachomatis [1,2]. The molecule of moxifloxacin binds to and inhibits the bacterial DNA gyrase, which is an essential enzyme that catalyzes the ATP-dependent negative super-coiling of double-stranded closed-circular DNA [3]. The blocking of DNA gyrase leads to the death of bacteria and prevents the worsening of infection.

The manufacture of drug substances consumes reactive reagents, starting materials, and intermediates [4]. Inevitably, there are impurities and associated byproducts that reside in final product [5]. One of the most widely used methods for producing moxifloxacin (Fig. 1a) is the condensation of 1-cyclopropyl-6,7-difluoro-1,4-dihydro-8-methoxy-4-oxo-3-quinolinecarboxylic acid and (4aS,7aS)-1H- octahydropyrrolo [3,4-b]pyridine (intermediate **1**) [6–8]. Intermediate **1** (Fig. 1b) is also known as (1S,6S)-2,8-diazabicyclo[4,3,0] nonane [9]. Naturally the intermediate **1** may reside in the final product as an impurity [10]. ICH M7 provides guidance on assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk [5,11]. The FDA and EMA both adopted the ICH-M7 as the guidance for industry. In the guidance of ICH-M7, the threshold of toxicological concern based on an acceptable intake of 1.5 µg/day is considered to be protective for a lifetime of daily exposure. Therefore, it is important to identify and quantify the intermediates of moxifloxacin.

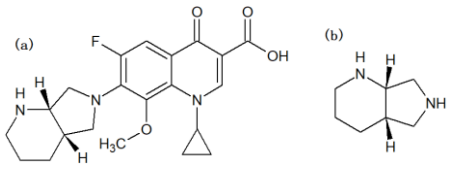

Fig. 1 Molecular structure of (a) moxifloxacin, (b) (4aS,7aS)-1H-octahydropyrrolo[3,4-b]pyridine (Intermediate **1**).

Five impurities of moxifloxacin are explicitly required to be tested according to the USP and EP. These five impurities are all byproducts of the moxifloxacin. Several analytical methods have been employed to determine moxifloxacin. Ultraviolet (UV) spectrophotometry has been used for the simultaneous determination of moxifloxacin and cefixime [12]. High-performance thin-layer chromatography (HPTLC) has also been applied for the degradation studies of moxifloxacin [13]. However, high-performance liquid chromatography (HPLC) [14,15] and its tandem techniques are the mostly used methods [16]. For example, Baoming Ning et al. have identified ten impurities by HPLC tandem with ultraviolet and Fourier transform ion cyclotron resonance mass spectrometry (HPLC–UV/FTICRMS) [17]. M.V Suryanarayana et al. detected four impurities in the synthesis product of moxifloxacin by a gradient HPLC method [18]. Jan Krzek et al. carried out an ultra HPLC-MS method to detect moxifloxacin and its oxidation products [19]. Also, there were reports that used LC–MS for the simultaneous analysis of moxifloxacin and other antibiotics in plasma [20–22], serum[23] and other matrices [24]. For example, Yi Hu et al. determined five antituberculosis drugs including moxifloxacin simultaneously in plasma by LC-MS/MS [25]. The fragmentation of patterns of

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moxifloxacin in MS/MS mode was investigated [26]. Besides LC methods, a fluorescence probe for the detection of moxifloxacin was also reported [27].

Despite the widely reported moxifloxacin analysis protocol, the detection method of intermediate **1** has received little attention. None of the aforementioned studies examined the intermediate **1**. Although intermediate **1** is a necessary intermediate, it is not listed as a moxifloxacin impurity that requiring mandatory inspection in USP or EP. The most likely reason is that after the salt formation reaction and recrystallization purification process, the intermediate **1** in the final product of moxifloxacin hydrochloride is expected to be negligible. Nonetheless, there is still this need for a practical analytical method to confirm it. More importantly, the detection of intermediate **1** content in the suspension solution during the moxifloxacin synthesis reaction is necessary.

The procedural approach for intermediate **1** check is thin layer chromatography (TLC) [13,28]. Traditional LC-UV cannot be used to analyze intermediate **1** directly since intermediate **1** does not have sufficient ultraviolet absorption. Consequently, it is impossible to analyze moxifloxacin and intermediate **1** simultaneously with LC-UV. However, a simultaneous and accurate method is needed for the manufacture of moxifloxacin during the process where the intermediate **1** and the product moxifloxacin coexist. Also, the concentration of moxifloxacin and its impurities in the final product should be determined. Gas chromatography (GC) is utilized for volatile and thermally stable compounds. The homologue of pyridine can be analyzed with gas chromatography. Unfortunately, as a pyridine derivative, intermediate **1** has a boiling point of 285.8 °C at 760 mmHg. It is not easy to analyze it with GC [29,30]. Alternatively, there were attempts to chemically derivatize the moxifloxacin and then analyze it by spectrophotometry [31]. Despite all this, there is no simple method for the simultaneous detection of two compounds.

To the best of our knowledge, there is no published study on the simultaneous detection of moxifloxacin and its intermediate **1**. In this study, the comprehensive applicability of the ultra-high performance LC (UHPLC) coupled with a high-resolution MS method was investigated for the simultaneous detection of moxifloxacin and intermediate **1**. The fast speed, high sensitivity, satisfied precision, and good specificity were achieved.

II. MATERIALS AND METHODS

A. *Chemicals and materials*

The reference substance of moxifloxacin hydrochloride (>99.5%) was acquired from Hairong Pharmaceutical Co., Ltd.(Nanjing, China). Intermediate 1 standard (>99.6%) was obtained from Haiheng Biochemical Technology Co., Ltd.(Lianyungang, China). Methanol and acetonitrile that purchased from Fisher Scientific (Fair Lawn, NJ, USA) were both of HPLC grade. Formic acid (>98%) was purchased from Merck (Darmstadt, Germany). Pure water was prepared with a water purifier (Milli-QDirect8, Millipore, France). Other reagents were of analytical grade unless otherwise specified.

B. *Instruments*

An Agilent 1290 infinity system (USA) was employed as a UHPLC device, which consisted of a binary pump, an autosampler, a column thermostat, and a DAD detector. The mass spectrometer was an AB Sciex TripleTOF 5600$^+$ (USA) with an ESI interface. Analytical balance (SartoriusBSA224S, China) and ultrasonic cleaner (KQ100DE, China) were used for sample preparation.

C. *Solution preparation*

A mixture of formic acid/methanol/water (0.1:50:50, v/v/v) was served as the dilution solution.

Concrete sample solution was obtained from the reactor that for moxifloxacin synthesis. When the condensation reaction of intermediate **1** and other precursor reactants was terminated, the suspension solutions of the products were accurately measured out immediately. The measured solutions were then quantitatively diluted with the dilution solution, agitated well and filtered through 0.22 μm microporous membranes.

To prepare stock standard solution, 20.0 mg of moxifloxacin hydrochloride were weighted and dissolved into 2 mL methanol with the aid of ultrasonic bath. The stock solution was then diluted step by step using the dilution solution to produce a series of standards solutions with equivalent moxifloxacin concentrations of 50 ng/mL, 100 ng/mL, 200 ng/mL, 300 ng/mL, 400 ng/mL and 500 ng/mL. The series of standards solutions were filtered through 0.22 μm microporous membranes. 20.0 mg intermediate **1** standard was dissolved into methanol to serve as stock solution. The stock solution was then diluted with the dilution solution gradually. The series standard solutions were prepared at the concentration of 5 ng/mL, 20 ng/mL, 40 ng/mL, 60 ng/mL, 80 ng/mL and 100 ng/mL respectively. All of them were filtered through 0.22 microporous membranes as well.

Two kinds of stock solutions were mixed and diluted with dilution solution to make a series of mixed standard solutions. The concentrations of moxifloxacin in each grade of mixed standard solution were 100 ng/mL, 200 ng/mL, 300 ng/mL, 400 ng/mL, and 500 ng/mL, whereas the concentrations of intermediate **1** were 10 ng/mL, 20 ng/mL, 30 ng/mL, 40 ng/mL, and 50 ng/mL respectively. The mixed standard solutions were also filtered with 0.22 μm microporous membranes for testing.

D. *UHPLC-MS condition*

The UHPLC separation was performed on a column of C18 (2.1×50 mm i.d., 1.7 μm particle size) (Waters Co., Milford, MA, USA). The mobile phase A consisted of a 0.05% (v/v) formic acid/water solution. The column temperature was set at 35 °C. The mobile phase B was methanol containing 0.05% (v/v) formic acid. The mobile phase flow rate was 0.2 mL/min. The injection volume was 1.0 μL. The ratio of phase A to phase B was adjusted according to the optimization progress. The isocratic elution with an equal phase A and phase B volume ratio (50%:50%) was finally selected as the optimized UHPLC condition.

The MS analysis was operated in ESI+ mode where the ion

source temperature was set at 550 ℃. The ionization voltage was set at +5 kV with 55 psi nebulizing gas, 55 psi auxiliary gas, and 35 psi curtain gas. The TOF scan range of m/z 105-1000 was recorded.

III. RESULTS AND DISCUSSION

A. *Optimization of the separation process*

In order to optimize the UHPLC-MS method, the mixed standard of 400 ng/mL moxifloxacin and 40 ng/mL intermediate **1** was analyzed as the model sample. Full gradient elution was carried out and, based on the results, the gradient program was optimized to a 50%:50% ratio of phase A and B from the elution beginning to end. At this constant composition of the mobile phase, the chromatographic separation process was completed within 2 min. As shown in the total ion flow (TIC) chromatogram (Fig. 2), the baseline separation was achieved for intermediate **1** and moxifloxacin. Under this isocratic condition, the retention time of intermediate **1** was about 0.67 min while the retention time of moxifloxacin was about 0.92 min. Both the peaks are in the good symmetrical shape. Therefore, the following experiments were all conducted based on this isocratic chromatographic method.

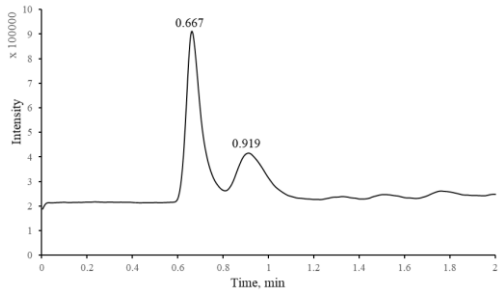

Fig. 2 The TIC of intermediate **1** and moxifloxacin under isocratic chromatographic condition

B. *Investigation of specificity*

The peak at retention time 0.667 min in TIC was assigned to intermediate **1**. Its specificity was confirmed by the retention time of the single standard sample. The mass spectrum at 0.667 min was shown in Fig. 3. The most abundance ion m/z 127.08 in the mass spectrum was assigned to the proton addition ion ($[M+H]^+$) of intermediate **1** (monoisotopic mass: 126.11). The m/z 127.08 ion signal was extracted from the TIC and the extracted ion chromatogram (EIC) was shown in Fig. 3 inset. In EIC, the retention time 0.69 min of intermediate **1** was reconfirmed. Also, the symmetrical peak and good signal-to-noise ratio (S/N) (>80:1) were observed. It implies that reliable quantification can be achieved with the peak integration in EIC.

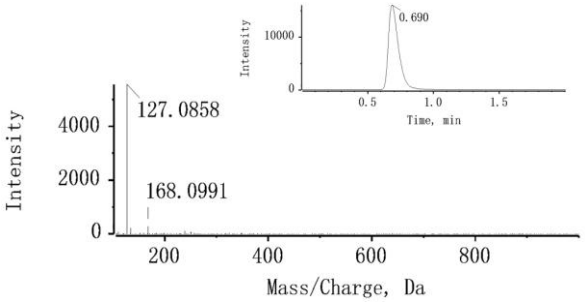

Fig. 3 The MS spectrum of intermediate **1** and its EIC (m/z 127.08).

The peak of 0.919 min in TIC was assigned to moxifloxacin. Its specificity was confirmed with the single standard of moxifloxacin. The mass spectrum at 0.919 min was shown in Fig. 4. The most abundance ion m/z 402.06 was assigned to the proton addition ion ([M+H]+) of moxifloxacin (monoisotopic mass: 401.17). The EIC of m/z 402.06 was shown in Fig. 4 inset, thereby the retention time 0.977 min of moxifloxacin was reconfirmed. The symmetrical peak and good S/N were observed as well.

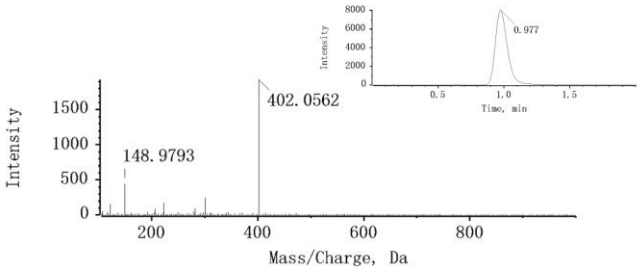

Fig. 4 The MS spectrum of moxifloxacin and its EIC (m/z 402.06).

The good peak outline and high S/N ratio of both analytes in each EIC indicate the satisfied specificity of the developed method.

C. *Method validation*

In order to evaluate the linear dynamic range of the developed method, a series of single control solutions was analyzed. For moxifloxacin, the solutions with concentrations of 100 ng/mL, 200 ng/mL, 300 ng/mL, 400 ng/mL and 500 ng/mL were analyzed sequentially. An aliquot of 1 μL of each standard solution was injected into the chromatographic instrument for UHPLC-MS analysis. The tests were performed in triplicate. The peak areas in the EIC of moxifloxacin were integrated. The regression of peak area versus sample concentration was illustrated in Fig. 5. The linear regression equation y=138.44x+1032.8 was calibrated along with the correlation coefficient R^2=0.9997. The good coefficient of concentration implies good sensitivity. The satisfied correlation coefficient indicates a good linear relationship over the range of 100-500 ng/mL for moxifloxacin.

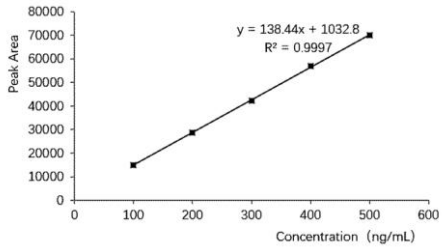

Fig. 5 The regression of peak area against concentration of moxifloxacin

The same investigation for intermediate **1** was carried out. The series of single standards were of 20 ng/mL, 40 ng/mL, 60 ng/mL, 80 ng/mL and 100 ng/mL. An aliquot of 1 μL of each standard sample was analyzed with the developed UHPLC-MS method. The analysis was repeated three times routinely. The peak areas in EIC versus concentration of intermediate **1** were illustrated in Fig. 6. The linear regression equation y=7459.4x+39265 was calculated and the correlation coefficient R^2=0.9983 was reached. In the range of 20-100 ng/mL of intermediate **1**, the developed method demonstrated the good linearity.

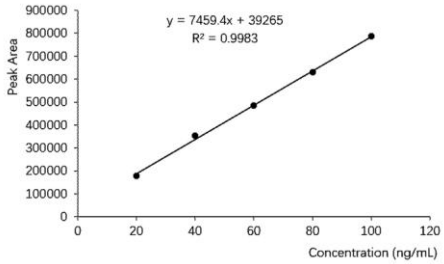

Fig. 6 The regression of peak area against concentration of intermediate **1**

The precision of the method was investigated with the mixed standard sample which containing 200 ng/mL moxifloxacin and 20 ng/mL intermediate **1**. Nine replicate experiments were carried out. The peak areas in each EIC for two compounds are all listed in Table 1 along with the average concentration and relative standard deviation (RSD) value. Both RSD values are less than 5%. It indicates that the precision of the method is reliable.

Table 1 Precision test results (peak area)

No.	moxifloxacin (200ng/mL)	intermediate **1** (20ng/mL)
1	27580.29849	177893.5685
2	30412.70597	171820.2474
3	28201.87595	187221.3639
4	27502.49387	186141.3124
5	27441.58664	173147.6874
6	29245.26626	168364.0503
7	29363.15055	184949.5901
8	30098.73848	167129.7408
9	28807.00557	185669.2168
Average	28739.23575	178037.4197
RSD(%)	3.91%	4.57%

The recovery of the method is the most commonly used parameter to indicate the accuracy. For method accuracy validation, the mixed standard sample that consisted of 300 ng/mL moxifloxacin and 30 ng/mL intermediate **1** was selected as the bulk sample. The bulk sample solution was added with both analytes. The added amount of analyte (C_{add}) in the final solution was controlled to be 30 ng/mL for moxifloxacin and 3 ng/mL for intermediate **1**. Both the spiked sample and the bulk sample were analyzed in 5 replicates with the developed UHPLC-MS method. The concentration of the bulk sample (C_{bulk}) and the concentration of the spiked sample (C_{spike}) were determined based on the peak area and the linear equation corresponding to each of the two analytes. The recovery for each of the analytes was calculated with the equation (1).

$$Recovery = (C_{spike} - C_{bulk})/C_{add} \times 100\% \quad (1)$$

The recovery results of moxifloxacin and intermediate **1** are listed in Table 2.

The average recovery of moxifloxacin is 97.10%. The value indicated that the method was accurate enough. The recovery deviation of intermediate **1** (91.03-104.94%) was greater than moxifloxacin (94.37-99.03%). Nevertheless, taking into account the relative low concentration level (3 ng/mL), the accuracy of the method for intermediate **1** is reliable. The RSD of 8.37% is acceptable.

Table 2 Recovery of moxifloxacin and intermediate **1**.

	No.	recovery	average recovery	RSD
Moxifloxacin (30 ng/mL)	1	95.48%	97.10%	2.21 %
	2	94.37%		
	3	99.23%		
	4	99.03%		
	5	97.38%		
intermediate **1** (3 ng/mL)	1	109.17 %	98.28%	8.37 %
	2	94.00%		
	3	92.24%		
	4	91.03%		
	5	104.94 %		

D. Examination of concrete samples

The typical concrete sample in this study was suspension solution which taken from the reactor. As soon as the moxifloxacin condensation process was terminated, the suspension solution was measured out. The concrete sample was then diluted into three concentration levels. Three level samples were all analyzed with the developed method.

For the moxifloxacin concentration determination, the peak area (RT 0.977 min) in the EIC (m/z 402.06) was integrated and substituted into the corresponding linear equation. The results of three level samples showed that the samples diluted on the order of 106 times were within the corresponding linear range (100-500 ng/mL). The determined value was 483.0 ng/mL for moxifloxacin. Therefore, the concentration of moxifloxacin in the original concrete sample was 483.0 g/L. As for intermediate **1**, the peak area (RT 0.69 min) in the EIC (m/z 127.08) was integrated and substituted into the corresponding linear equation. The results showed that the samples diluted on the order of 105 times were within the

corresponding linear range (20-100 ng/mL). The determined value was 52.16 ng/mL for intermediate **1**. Thus, the concentration of intermediate **1** in the original sample was 5.216 g/L. The concentration results of concrete samples showed that further purification for the final moxifloxacin product is necessary.

IV. Conclusion

For the first time, moxifloxacin and its intermediate **1** were detected concurrently. A whole UHPLC-MS method was developed for this objective. The whole chromatographic separation was accomplished in 2 minutes under the isocratic elution condition. The retention time of intermediate **1** was approximately 0.67 min, while that of moxifloxacin was approximately 0.92 min. Both the peaks are in good symmetrical shape. The specificity of the method was confirmed by the retention time of the single standard sample. Also, the proton addition ion ([M+H]+) of each analyte was confirmed in their corresponding MS spectra. The method validation showed that the developed method was reliable, precise, and accurate. Based on the peak area in the EIC, the linear regressions were calibrated for both compounds. The correlation coefficient R^2=0.9997 indicates a good linear relationship over the range of 100-500 ng/mL for moxifloxacin. The dynamic range of intermediate **1** was 20-100 ng/mL (R^2=0.9983). The relative standard deviation values (n=9) were both less than 5% for two analytes in the mixed standard sample. The average recovery of moxifloxacin at 30 ng/mL was 97.10%, while the average recovery of intermediate **1** at 3 ng/mL was 98.28%. A typical concrete sample during moxifloxacin synthesis process was analyzed. The intermediate **1** in the suspension of the concrete sample was 5.216 g/L. This non-negligible concentration indicates that further purification is necessary. Overall, this rapid, sensitive, accurate, and highly specific method is suitable for the process monitoring and the final quality control during the moxifloxacin manufacture.

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