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Determination of moxifloxacin in human plasma by liquid chromatography electrospray ionization tandem mass spectrometry

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Abstract

Moxifloxacin is an advanced-generation, 8-methoxy fluoroquinolone that is active against a broad spectrum of pathogens, including antibiotic resistant *Streptococcus pneumoniae*. Development of a rapid, sensitive and selective method for the determination of moxifloxacin in human plasma is essential for understanding the pharmacokinetics of the drug when administered orally or intravenously. Solid phase extraction (SPE) using Oasis® HLB was used to extract moxifloxacin and the internal standard lomefloxacin from plasma. A method based on liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS) was developed and validated to quantitate moxifloxacin in human plasma. The precursor and major product ion of the analyte was monitored on a triple quadrupole mass spectrometer with positive ion electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode. Mechanisms for the formation of collision-induced dissociation (CID) products of moxifloxacin are proposed. Linear calibration curves were generated from 1 to 1000 ng/ml with coefficients of determination greater than 0.999. The inter-day and intra-day precision (% CV) was less than 11.3% and accuracy (% error) was less than 10.0% for moxifloxacin. The limit of detection (LOD) for the method was 50 pg/ml based on a signal to noise ratio of 3.

Keywords: Moxifloxacin; 8-Methoxy fluoroquinolone; Liquid chromatography/tandem mass spectrometry; Electrospray ionization; Multiple reaction monitoring

1. Introduction

Respiratory infections are a major cause of morbidity in the US [1]. Various antibiotics used to treat these infections include penicillins, cephalosporins, macrolide antibiotics, tetracyclines and quinolones [1]. The widespread use of earlier generation of quinolones has led to pathogen resistance and treatment failures, especially with isolates of *Streptococcus pneumoniae*. Moxifloxacin {1-cyclopropyl-7-(2,8-diazobicyclo[4.3.0]nonane)-6-fluoro-8-methoxy-1,4-dihydro-4-oxo-3-quinolone carboxylic acid} is a new fourth generation quinolone with demonstrated effectiveness

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against acute bacterial sinusitis, acute bacterial exacerbation of chronic bronchitis, and community-acquired pneumonia [2–8]. In addition to enhanced activity against many clinical isolates, moxifloxacin has been chemically engineered to optimize its safety and pharmacokinetic profiles. The absence of a halide at the C8 position minimizes the potential for photosensitivity reactions, and a methoxy group at that position theoretically may confer enhanced activity against resistant gram-positive bacteria and reduced selection of resistant mutants [9,10]. Moxifloxacin also has a diazobicyclononyl ring moiety at the 7 position, which enhances the potency, spectrum of activity and half-life [11]. The drug is rapidly absorbed, with peak plasma concentrations reached within 1–4 h after treatment and a long half-life (11.4–15.6 h) making it suitable for once-daily administration. Moxifloxacin appears promising for the treatment of respiratory tract infections caused by common bacterial species [12].

Liquid chromatography is widely used for the quantitative determination of pharmaceutical compounds with UV, fluorescence or electrochemical detection. For the determination of pharmaceutical compounds for clinical and pre-clinical studies, LC/MS with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APcI) is established as a powerful analytical tool [13–17].

Quinolones are normally quantitated by reversed-phase high performance liquid chromatography (RP-HPLC) with UV detection or fluorometric detection [18]. Only three papers concerning the use of HPLC [19–21] and capillary electrophoresis [22] for moxifloxacin determination have been published. Recently, a spectrofluorometric method was reported for the determination of moxifloxacin in human serum and urine [23]. There has not been a paper published on the determination of moxifloxacin using LC–MS/MS. In this paper, an LC/ESI–MS/MS method was developed to analyze moxifloxacin in human plasma using solid phase extraction (SPE) as a sample pretreatment procedure.

ESI tandem mass spectrometry (MS/MS) provides the analyst with a rugged, sensitive and widely used technique to mass select a precursor and a characteristic product ion of an analyte, making it a highly specific method for the analysis of drugs in human plasma. In the present study, SPE and online LC/ESI–MS/MS using lomefloxacin as an internal standard was utilized to quantitate moxifloxacin in human plasma in less than 4 min with a LOQ of 1 ng/ml is described. Collision-induced dissociation (CID) data on the compounds are presented and mechanisms for the formation of the observed product ions are proposed.

2. Experimental

2.1. Materials

Moxifloxacin (Bay 12-8039, Batch 502610, Fig. 1) was a gift from Bayer Pharmaceutical Division (West Haven, CT 06516). Lomefloxacin (internal standard, Fig. 1) was obtained from Sigma (St. Louis, MO 63195). Methanol (HPLC grade) and formic acid (88%) were obtained from J.T. Baker (Phillipsburg, NJ 08865). Deionized water was purified by a cartridge system (Continental Water Systems, Roswell, GA 30076). Trifluoroacetic acid (99%) was obtained from Aldrich Chemical Company (Milwaukee, WI 55325). Oasis® HLB solid phase cartridges were obtained from Waters Corporation (Milford, MA 01757).

2.2. Preparation of stock and sample solutions

Stock solutions of moxifloxacin and lomefloxacin were prepared at a concentration of 100 μg/ml in distilled water. A working internal standard

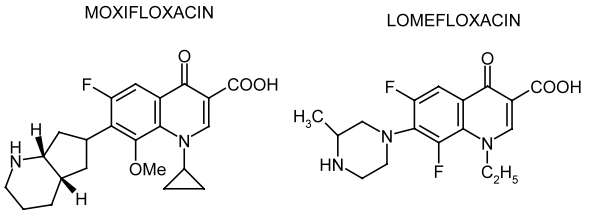

Fig. 1. Structural formulae of moxifloxacin and lomefloxacin.

solution was prepared by diluting the lomefloxacin stock solution with distilled water to provide a concentration of 500 ng/ml. An intermediate stock solution of 10 μg/ml containing moxifloxacin was prepared by diluting the original stock solution with distilled water. For preparation of standard curves, seven dilutions containing moxifloxacin were prepared at 1, 2, 10, 20, 100, 200 and 1000 ng/ml in blank plasma. QC samples were prepared at concentrations of 4, 40 and 400 ng/ml in blank plasma and were stored at −20 °C until assayed or used for validating the analytical method.

2.3. Solid phase extraction procedure

Extraction of the analytes was based on the modifications of a method previously reported by Lemoine et al. [20]. One millilitre of each calibration standard and QC samples were thawed and mixed well with a vortex mixer. To each sample was added 100 μl of 500 ng/ml internal standard solution in a glass tube and mixed well. Oasis® HLB SPE cartridges (30 mg, 1 cc) were conditioned with 1 ml methanol and 1ml deionized water. The samples were added to the cartridges and vacuum was applied. The cartridges were washed with 2 × 1 ml water. The sample was eluted using 1 ml of 99.9:0.1 v/v methanol–TFA followed by sample concentration in a vacuum centrifuge (Savant Instruments Inc., Farmingdale, NY). Extracts were then reconstituted in 100 μl of mobile phase, mixed, and 10 μl injected into the LC/ESI–MS/MS system.

2.4. LC/ESI–MS/MS conditions

The LC separation was performed using two Perkin–Elmer Series 200 micropumps and a Perkin–Elmer Series 200 Autosampler (MDS Sciex, Concord, Ont., Canada). The column utilized was a BDS Hypersil C18 column (100 × 4.6 mm i.d., 5 μm particle size, ThermoHypersil Keystone, Bellefonte, PA). The flow rate was 1000 μl/min through the LC column which was split 1:5 using a diverter (Accurate) from LC packings (San Franciso, CA) prior to introduction into the Turboionspray interface of the mass spectrometer. Ten microlitres of injections of the reconstituted extracts were injected onto the HPLC system for analysis. An isocratic mobile phase of 60: 40 v/v 0.1% formic acid–acetonitrile was used and the assay run time was less than 4.0 min A PE Sciex API 3000 operating in the Turboionspray mode was used for detection of the positive ions (Concord, Ont., Canada). The measurements were made at a 400 °C source temperature, 3500 V ionspray voltage, 65 V declustering potential, 300 V focusing potential and 20 eV collision energy with gas 1 and 2 set at 45 and 80 (arbitrary units), respectively. The nitrogen collision gas setting was six (arbitrary units). The assay was based on monitoring the $[M+H]^+$ ions for the analyte and the internal standard in the first quadrupole and their corresponding product ions in the third quadrupole with a dwell time of 100 ms. Selected reaction monitoring (SRM) chromatographic data was collected using SCIEX ANALYST® 1.1 software.

2.5. Calibration and calculation procedures

Daily calibration curves were constructed using the ratios of the observed peak areas of moxifloxacin and the internal standard. Unknown concentrations were computed from the $1/x$ weighted linear regression equation of the peak area ratio against concentration for the calibration curve.

2.6. Accuracy and precision

The intra-day accuracy and precision of the method were estimated from the back-calculated standard concentrations. The overall mean precision was defined by the relative standard deviation (R.S.D.) with relative errors from seven standards and three QCs analyzed on the same day ($n = 6$). Inter-day variability was estimated from the analyses of six replicates of standards and QC samples at three levels on three separate days ($n = 18$ per level).

3. Results and discussion

We have developed a specific LC–MS/MS assay to determine moxifloxacin from human plasma with a LOD of 50 pg/ml and with a run time of less

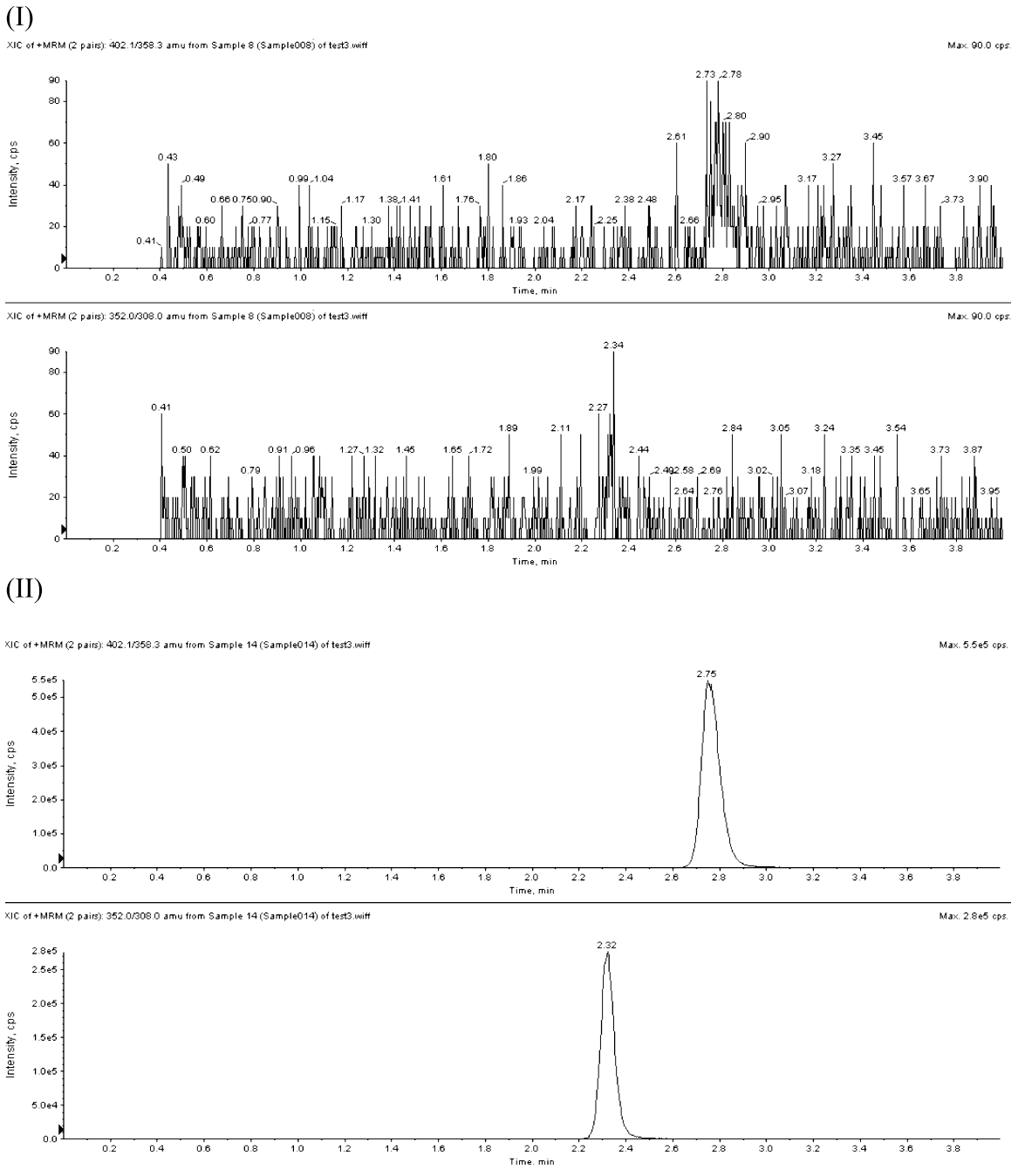

Fig. 2. Typical chromatograms of (I) blank human plasma and (II) blank human plasma spiked with moxifloxacin at 100 ng/ml (2.75 min) and the internal standard, lomefloxacin (2.32 min) at 500 ng/ml.

than 4.0 min. The previous method described by Lemoine et al. [20] had a LOD of 6.5 ng/ml and a run time of more than 12 min per injection. Another method described by Stass and Dalhoff [19] used protein precipitation with acetonitrile as the sample clean-up procedure and had a LOQ of

Table 1
m/z Ratios and relative intensities from positive ion MS/MS spectra

Moxifloxacin (%)[a]	Lomefloxacin (%)[a]
402 (100)	352 (100)
384 (10)	334 (30)
358(10)	308 (30)

[a] Experiments performed at collision energy of 20 eV. See Section 2 for other conditions.

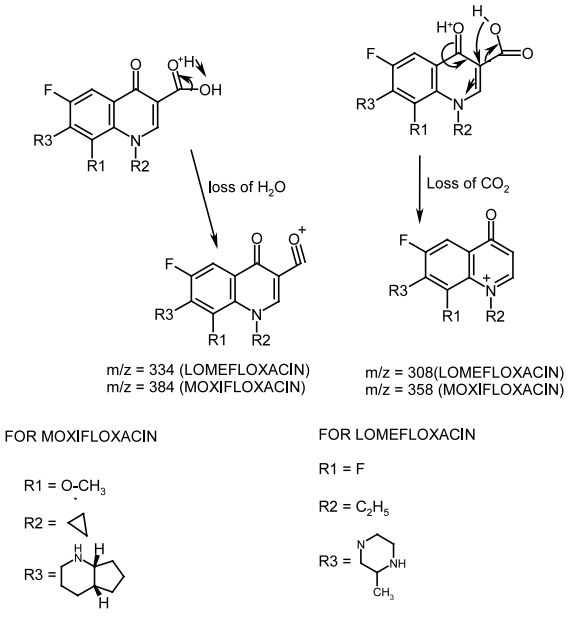

Fig. 3. Fragmentation pathway for moxifloxacin and lomefloxacin.

2.5 ng/ml with a run time of 8–10 min, depending on the matrix. A recent method by Ba and coworkers uses HPLC and fluorescence detection to quantitate moxifloxacin from growth media [21]. This method has an LOQ of 50 ng/ml and a run time of 6 min. The procedure described in this paper uses SPE for an efficient extraction of moxifloxacin and the internal standard in a relatively short time with excellent recovery. Extraction recoveries ($n = 5$) were determined by external standard comparison and were approximately, $90.6 \pm 2.8\%$ at 10 and $92.7 \pm 0.7\%$ at 200 ng/ml for moxifloxacin and $97.4 \pm 1.5\%$ for the internal standard lomefloxacin at 500 ng/ml, in plasma. It was determined that the most efficient extraction was performed using Oasis® HLB cartridges at the conditions described in the extraction procedure (see Section 2). Other cartridges studied were C18, C8, C2 and silica, but either the recoveries were low ($< 60\%$) or interferences were observed. The Oasis® HLB cartridge yielded good reproducible recoveries for moxifloxacin and the internal standard. The extracts were also free of particulate matter and endogenous interferences. Hence, Oasis® HLB cartridges were used for the extraction of moxifloxacin.

For quantitative LC/ESI–MS/MS, the positive ionization mode was selected because of improved sensitivity due to the presence of amine and ketone groups, which were easily protonated. Representative chromatograms of the blank human plasma and blank human plasma spiked with moxifloxacin (100 ng/ml) and the internal standard lomefloxacin (500 ng/ml) are shown in Fig. 2.

Table 2
Calibration curve parameters for moxifloxacin

Run number	Slope	Intercept	*R*-squared	LOQ	ULQ
Day 1	0.000159536	0.000264442	0.9992	1.0	1000.0
Day 2	0.000158972	0.000344964	0.9996	1.0	1000.0
Day 3	0.000159306	0.000322703	0.9993	1.0	1000.0
Mean	0.000159271	0.000310703	0.9994		
S.D.	0.000000284	0.000041581	0.0002		
%R.S.D.	0.18	13.40	0.02		
N	18	18	18		

Table 3
Inter-day accuracy and precision data for spiked samples

	Std 1 1 (ng/ml)	Std 2 2 (ng/ml)	QC 1 4.0 (ng/ml)	Std 3 10.0 (ng/ml)	Std 4 20.0 (ng/ml)	QC 2 40.0 (ng/ml)	Std 5 100.0 (ng/ml)	Std 6 200.0 (ng/ml)	QC 3 400.0 (ng/ml)	Std 7 1000.0 (ng/ml)
Day 1	1.2	2.1	4.0	10.6	19.5	39.4	103.3	195.5	412.3	1038.3
	1.0	1.8	3.5	9.6	20.1	39.9	104.4	200.5	407.9	961.5
	1.1	1.6	3.7	8.8	19.7	39.9	100.2	194.8	414.9	969.7
	1.3	2.5	4.1	9.1	19.4	40.4	100.3	204.6	383.9	978.4
	1.3	1.7	3.6	9.8	19.0	38.0	103.1	192.4	398.3	1028.5
	1.0	1.9	3.7	8.6	19.3	37.1	97.7	199.3	404.4	1018.4
Day 2	1.0	2.0	3.8	9.4	20.0	43.5	100.1	194.4	390.5	976.2
	0.9	2.0	4.2	9.2	19.8	40.2	101.5	208.4	401.6	1013.2
	1.1	2.1	4.1	9.9	20.1	40.9	99.0	202.8	402.6	1007.3
	1.1	2.2	3.6	8.9	18.5	39.4	98.9	203.7	380.6	1032.3
	1.1	1.9	3.8	10.1	19.8	37.5	98.5	197.8	408.1	992.7
	1.1	2.1	4.5	10.3	18.3	38.0	98.0	202.5	409.8	985.9
Day 3	1.2	2.1	3.6	10.1	18.8	39.2	99.4	196.5	402.6	997.3
	0.9	2.2	3.8	10.2	19.6	38.6	99.0	198.7	411.5	964.3
	0.9	1.9	3.9	9.3	19.4	39.4	101.2	202.4	406.2	958.3
	1.0	2.0	4.2	9.6	19.6	40.4	100.3	206.5	399.7	987.6
	1.1	2.1	3.9	9.5	20.1	40.5	98.6	199.1	400.1	973.3
	1.1	1.7	3.8	10.5	20.4	38.4	102.4	197.6	396.7	999.4
Mean	1.1	2.0	3.9	9.6	19.5	39.5	100.3	199.9	401.8	993.5
S.D.	0.1	0.2	0.3	0.6	0.6	1.5	2.0	4.4	9.4	25.1
% R.S.D.	11.3	10.8	6.8	6.1	2.9	3.8	1.9	2.2	2.3	2.5
% Bias	10	0	−2.5	−4	−2.5	−1.3	0.3	−0.05	0.5	0.7
n	18	18	18	18	18	18	18	18	18	18

*, Mean ± S.D.; based on $n = 18$.

3.1. Mass spectra and total ion chromatograms

In all analytes, the precursor ion $[M+H]^+$, where M is the molecular mass of the respective analyte, is formed as a result of the addition of a proton to form the positively charged molecular ion. The positive ion tandem mass spectral data of moxifloxacin, as well as the internal standard lomefloxacin, are shown in Table 1. The base peak in the CID mass spectrum is m/z 402 for moxifloxacin and m/z 352 for lomefloxacin. Increasing the collision energy to optimize the formation of product ions resulted in numerous fragment ions and did not improve the sensitivity of the assay. The precursor and major product ions of the analytes were monitored in the multiple reaction mode as follows: (1) moxifloxacin (m/z $402 \rightarrow 384$), (2) lomefloxacin (m/z $352 \rightarrow 308$). The formation of the product ions occur either by the loss of H_2O (m/z 334 for lomefloxacin and 384 for moxifloxacin) or by the loss of CO_2 (m/z 358 for moxifloxacin and 308 for lomefloxacin). Mechanisms for the formation of these ions are shown in Fig. 3.

3.2. Linear regression and detection limits

Standard curves were produced on three different days in spiked human plasma over the range of 1–1000 ng/ml, encompassing the therapeutic range of moxifloxacin. The response was linear in this range and correlation coefficient (r) and the coefficient of determination (r^2) were greater than 0.99. The limit of detection (LOD) for moxifloxacin in human plasma, based on a signal to noise ratio of three was 50 pg/ml. The calibration curve parameters are given in Table 2.

3.3. Intra- and inter-day precision and accuracy

Intra- ($n = 6$) and inter-day ($n = 18$) precision and accuracy calculated from QC samples analyzed on 3 days for moxifloxacin at concentrations of 4, 40 and 400 ng/ml are tabulated in Table 3. The inter-day and intra-day precision (% R.S.D.) was less than 11.3% and accuracy (% error) was less than 10.0% for moxifloxacin.

4. Conclusions

SPE and LC/ESI–MS/MS provide the analyst with a novel method to determine levels of moxifloxacin in human plasma. The described method will generate quantitative extractions without the need for excessive sample cleanup steps. LC/ESI–MS/MS, in addition to being fast (run time less than 4.0 min) specific, rugged and easy to use method, it provides sensitivity in the low ng/ml range. The major advantages of this method are the rapid separation and the specific detection used.

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