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THERAPEUTIC MONITORING OF LEVOFLOXACIN: A NEW LC-MS/MS METHOD FOR QUANTIFICATION OF LEVOFLOXACIN IN HUMAN PLASMA

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ABSTRACT. A simple and sensitive liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method for the quantification of levofloxacin in human plasma was developed and validated. The separation was performed on a Zorbax SB-C18 column under isocratic conditions using a mobile phase of 17:83 (v/v) acetonitrile and 0.1% (v/v) formic acid in water at 50ºC with a flow rate of 1 mL/min. The detection of levofloxacin was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer with electrospray positive ionisation. The human plasma samples (0.1 mL) were deproteinised with methanol and aliquots of 1 µL from supernatants obtained after centrifugation were directly injected into the chromatographic system. The method shows a good linearity (r^2 > 0.99), precision (CV < 11%) and accuracy (bias < 4.7%) over the range of 0.1-10.0 µg/mL plasma. The lower limit of quantification (LLOQ) was 0.1 µg/mL and the recovery was between 95.2-104.5%. The method is not expensive, it needs a minimum time for plasma sample preparation and has a run-time of 1.3 min for instrument analysis (retention time of levofloxacin was 0.9 min). The developed and validated method is very simple, rapid and efficient, with wide applications in clinical level monitoring, pharmacokinetics and bioequivalence studies of levofloxacin.

Keywords: *levofloxacin, LC-MS/MS, therapeutic drug monitoring*

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INTRODUCTION

Levofloxacin, (-)-(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid (Fig.1), is a systemic drug from third generation of quinolones [1]. It is the active S-(-) enantiomer of ofloxacin and has bactericidal activity against a broad spectrum of gram-negative and gram-positive aerobes and atypical bacteria. It has limited activity against anaerobes [2]. Its therapeutic effectiveness is due to its capacity to inhibit two enzymes involved in bacterial DNA replication: the DNA gyrases and topoisomerase IV. Thus, levofloxacin affects DNA replication, transcription, repair and recombination. The effect is initially bacteriostatic but becomes bactericidal when bacteria are unable to repair the DNA lesions [Modern]. Therapeutic use of levofloxacin includes mainly urinary and respiratory tract infections, as well as systemic infections [3, 4].

The antibiotic activity of fluoroquinolones depends on the ratio of maximum drug concentration (Cmax) to minimum inhibitory concentration (MIC). Moreover, the ratio of the 24 h area under the concentration-time curve (AUC_{24}) of fluoroquinolones to MIC is an important predictor of treatment efficacy [5]. Therefore to have an effective dosage and to prevent bacterial resistance the monitoring of plasma concentrations of fluoroquinolones is recommended.

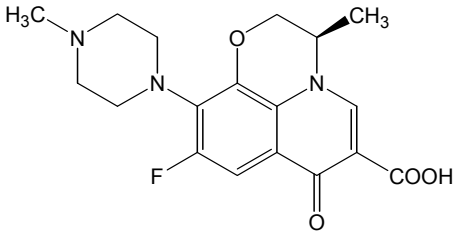

Figure 1. Chemical structure of levofloxacin

Levofloxacin is rapidly absorbed from the digestive tract and its oral bioavailability is ~99%. The peak plasma levels occur in 1-2 h. The therapeutic plasma concentrations are usually in the range of 0.5 - 6 μg/mL after oral administration or perfusion; Cmax can grow up to 12 μg/mL after high doses of levofloxacin. The plasma protein binding is between 30-40% and the drug is widely distributed in body tissues. Levofloxacin is excreted in the urine almost unchanged (80-85%) with a plasma elimination half-life of 6-8 h, being increased in renal impairment [1-3,6,7].

Several methods involving quantitative nuclear magnetic resonance spectrometry [8], capillary electrophoresis [9], high-performance thin-layer chromatography (HPTLC) [10] and high-performance liquid-chromatography (HPLC) with UV [11-16], fluorescence [5, 17-20] or mass spectrometric (MS)

[6, 21-23] detection have been reported to determine therapeutic levels of levofloxacin or ofloxacin in biological samples: serum [5] or plasma [8, 10, 14-23], urine [8, 14, 17], tissues [6, 22].

Liquid chromatography coupled with mass spectrometry has become increasingly popular in recent years, taking the place of conventional HPLC methods with UV, fluorescence or electrochemical detection due to its powerful performances. It is more rapid, usually requires a simple pre-treatment of samples, and offers an extraordinary selectivity, sensitivity and robustness [24-29]. The combination of HPLC with tandem mass spectrometry is becoming the method of choice for therapeutic drug monitoring and toxicology studies [30].

The aim of this study was to develop and validate a new simple and efficient LC/MS/MS assay for the quantification of levofloxacin in human plasma. This method will be applied in therapeutic drug monitoring, as well as in pharmacokinetics or bioavailability studies.

RESULTS AND DISCUSSION

The developed LC/MS-MS method was optimized and validated. It is rapid, with a total run time of instrumental analysis of 1.3 min and a retention time of levofloxacin of 0.9 min (Fig. 2). Sample preparation consisted only of protein precipitation. The volume of plasma required for processing was small, of 0.1 mL. All these features make the method ideal for routine analysis.

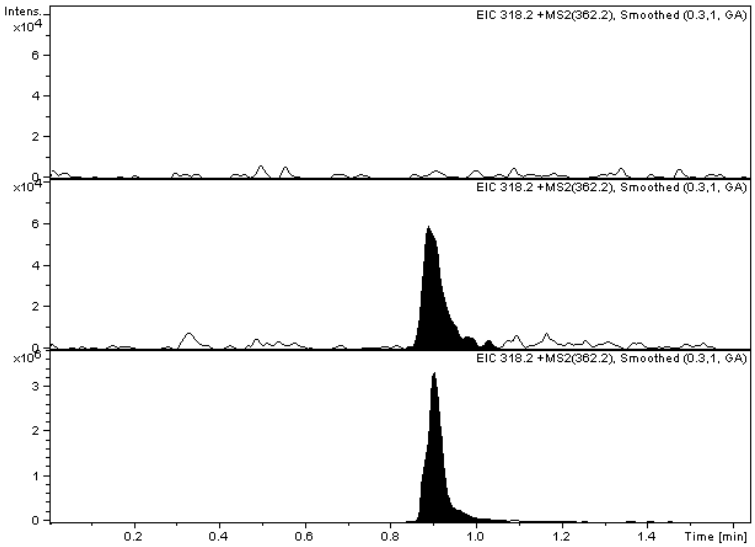

Figure 2. Representative chromatograms of (up) drug-free plasma, (middle) plasma spiked with levofloxacin at lower limit of quantification (0.1 μg/mL) and (down) plasma sample obtained from a patient 4 h after administration of 500 mg levofloxacin in perfusion (concentration found: 4.18 μg/mL).

The sensitivity of the developed method was good (LLOQ of 100 ng/mL), sufficient to determine therapeutic levels of levofloxacin, which are greater than 0.5 µg/mL. The absolute recoveries were high (between 91.4-100.1% at LLOQ, and 86.6-103.8% at 3.200 µg/mL, respectively).

Sample preparation

The assay sensitivity depends primarily on detection mode, but the method involved in sample preparation may also influence the chromatographic background level and can generate matrix suppression effect in LC-MS assays. An extraction step in plasma sample preparation to eliminate the impurities and to enhance sensitivity increases the time of analysis and the costs and can affect the recovery. In the method elaborated by Ji *et al.,* levofloxacin was isolated from plasma by extraction in dichloromethane, but even if the limit of quantification (LOQ) was 10 ng/mL, the recovery mean was only 55.5% [21]. In scientific literature, there are some LC-MS/MS methods that use precipitation of proteins (with acetonitrile or methanol) without extraction for the determination of levofloxacin in human plasma with better recoveries, > 70% (Table 1).

LC-MS/MS assay

The chromatographic conditions, especially the composition of mobile phase, were optimized in several trials to achieve maximum peak responses and symmetrical chromatographic peaks, a short retention time of levofloxacin and consequently a shorter run time of analysis. The best results were obtained with the mixture of acetonitrile and 0.1% (v/v) formic acid in water (17:83, v/v) under isocratic conditions.

In the case of levofloxacin, electrospray ionization (ESI) mode offers significantly higher signals compared to atmospheric pressure chemical ionization (APCI). The signal intensities of levofloxacin obtained in positive ion mode were much higher than those in negative ion mode, so the former ionization mode was chosen.

The direct MS detection is used for pharmaceutical purposes in qualitative rather than quantitative analysis. The use of tandem MS detection allows the obtention of better selectivity and sensitivity by the fragmentation of the molecular ion into several ions. After the collision that induced the dissociation of levofloxacin in ion trap mass spectrometer, the molecular ion $[M+H]^+$ (m/z 362.2) produced one abundant ion (m/z 318.2) $[M,H\text{-}CO_2]$ at the optimum collision energy of 1.2V (Fig. 3), thus, the detection of levofloxacin was carried out in multiple reaction monitoring (MRM) by monitoring the transition m/z 362.2 → m/z 318.2. No matrix interference or ion suppression was observed from the plasma samples.

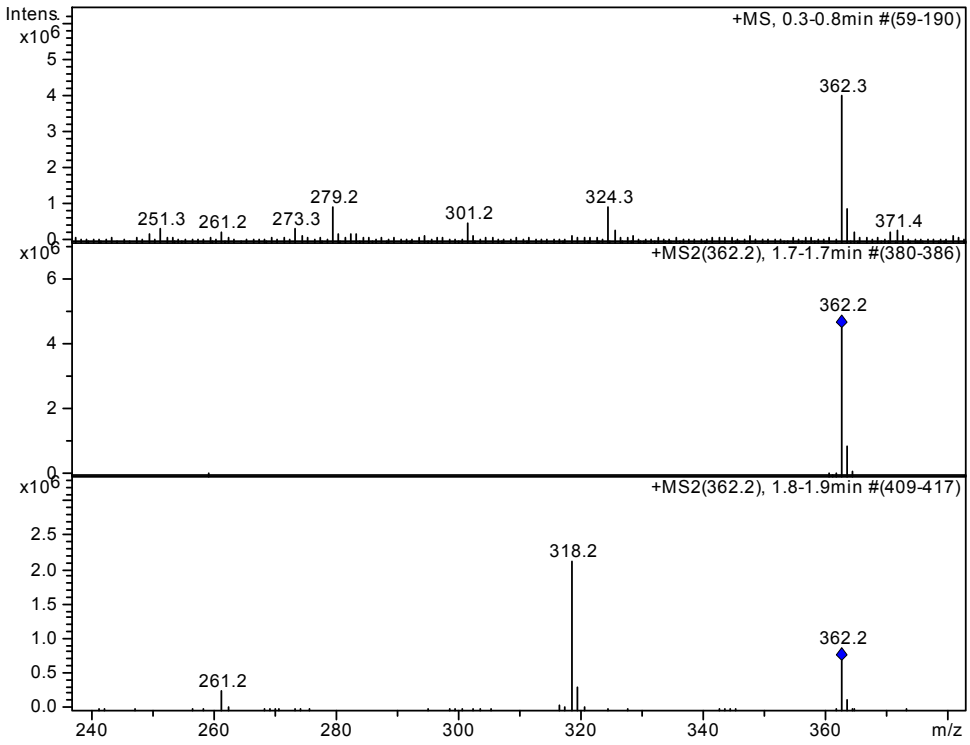

Figure 3. Mass spectra of levofloxacin obtained by electrospray ionisation in positive ion mode at the collision energy of 1.2V: (up) full-scan spectrum; (middle) MS spectrum of pseudo-molecular ion $[M+H]^+$; (down) MS/MS reactive spectrum (after fragmentation; monitored ion: m/z 318).

Other similar methods that operated in ESI(+)-MRM mode using the same transition monitoring were reported in the literature to quantify levofloxacin in plasma [22, 23] or other various samples as tissues samples [6], environmental water and swine wastewater [31, 32]. Of all these methods, those applied to plasma showed better sensitivity (LOQ < 100 ng/mL) using small volumes of plasma [22, 23] (Table 1) compared to that developed by us. However, our method is more rapid and has the characteristics of a high-throughput assay. It offers a shorter time of analysis and a lower cost in the case of routine measurements as compared to the other longer methods reported in literature (Table 1).

As the therapeutic plasma levels of levofloxacin are between 0.5-6 µg/mL, the LLOQ of 0.100 µg/mL established in our method can be accepted in bioequivalence studies and for routine purposes in therapeutic level monitoring of levofloxacin in human plasma.

Table 1. Analytical characteristics of some reported HPLC methods for the determination of levofloxacin or ofloxacin in human plasma or serum

Ref.	Matrix (mL)	Pre-treatment/ extraction[c]	Stationary phase	Mobile phase constituents[b]	Detection mode[a]	LOQ[d] (ng/mL)	Rt[e] (min)	Absolute recovery (%)
Our method	Plasma (0.1)	PP with methanol	Zorbax SB-C18	ACN: 0.1% (v/v) formic acid (17:83,v/v)	ESI-MS/MS, MRM (m/z 362.2 →318.2)	100	0.9	95.2-104.5
Ji [21]	Plasma (0.02)	ELL	HILIC Silica	ACN-100mM ammonium formate (pH 6.5) (82:18, v/v)	MS/MS, ESI, MRM (m/z 362.7→261.2)	10	1.9	55.2
Fang [22]	Plasma (0.15)	PP with MeOH	C4	MeOH-0.05% formic acid in water, gradient	ESI-MS/MS, MRM (m/z 362.1→318.1)	21.8	10.0	81.9-99.1
Meredith [23]	Plasma (0.02)	PP with ACN and MeOH	Phenomenex Luna, PFP	ACN-0.1% formic acid, gradient	ESI-MS/MS, MRM (m/z 362.1→318.3)	78	3.2	>70
Watabe [5]	Serum (0.20)	PP with $HClO_4$ and MeOH	Inertsil C8	ACN-1% TEA (pH 3) (14:86, v/v)	HPLC-FD	100	12.8	86.9-91.4
Wagen-lehner [17]	Serum (NA)	PP with ACN and $HClO_4$	Reversed phase	Citric acid buffer + ammonium perchlorate – ACN + ion pairing reagent (90:10,v/v)	HPLC-FD	2.34	NA[f]	NA
Tsaganos [19]	Plasma (0.50)	PP with ACN and trichloro-acetic acid	Nucleosil C18	25 mM sodium phosphate buffer (pH 3), 10 mM SDS: ACN (35:65, v/v)	HPLC-FD	390	2.9	NA
Zhou [20]	Plasma (0.1)	LLE	Kromasil C18	10mM phosphate buffer, pH 3.0 (with 0.01% TEA):ACN (76:24, v/v)	HPLC-FD	52.1	2.5	~86%
Siewert [18]	Plasma (0.05)	PP with trifluoro-acetic acid	YMC Pro C18	MeOH / 1.0 M ammonium acetate / H2O, gradient	HPLC-UV	100	8.3	97.2-104.7
Wong [14]	Plasma (NA)	LLE	Inertsil C18	NA (containing chiral reagents)	HPLC-UV	82	NA	NA
Kumar [15]	Plasma (0.5)	LLE	C18	20mM KH2PO4 buffer, pH 2.5:CAN (80:20, v/v)	HPLC-UV	100	5.9	~85%
Gao [16]	Plasma (0.5)	PP with HClO4	Kromasil C18	ACN:H2O:H3PO4: TEA (14:86:0.6:0.3, v/v/v/v)	HPLC-UV	50	8.4	89-98

[a] MRM, multiple reaction monitoring; FD, fluorescence detection; [b] MeOH, methanol; ACN, acetonitrile; TEA, triethylamine; [c] PP, protein precipitation; LLE, liquid-liquid extraction; [d] LOQ, limit of quantification; [e] Rt, retention time; [f] NA, not available.

Assay validation

The method was validated in accordance with international regulations [33, 34]. Representative chromatograms of drug-free plasma and plasma spiked with levofloxacin at LLOQ are shown in Fig. 2. No interfering peaks from the endogenous plasma components were observed in the retention time of levofloxacin.

The calibration curves were linear over the concentration range of 0.100 – 10 μg/mL in human plasma, with a correlation coefficient greater than 0.99. The LLOQ was 0.100 μg/mL. The values obtained for intra-day and inter-day precision and accuracy during the validation are shown in Tables 2 and 3, respectively.

All values for accuracy and precision were within recommended limits (<15%). The means of absolute recovery values were between 95% and 104.5%.

Method application

The validated method was used for therapeutic drug monitoring of levofloxacin (Fig. 2). As well other quinolones, levofloxacin has an excellent tissue and tissue fluid penetration, so it can be used for treatment of infections in a wide range of organ systems. It is available in both oral and intravenous formulations and one of its major advantages is the ability to treat many serious infections with oral or intravenous-oral switch regimens. Levofloxacin is used in treatment of urinary tract infections (pyelonefritis and complicated urinary tract infections, chronic bacterial prostatitis), respiratory tract infections (acute sinusitis, acute bacterial exacerbation of chronic bronchitis, community acquired pneumonia, skin and skin structure infection and post exposure prophylaxis and curative treatment after Anthrax inhalation (but treating physicians should refer to national consensus documents regarding the treatment of Bacillus anthracis).

Table 2. The intra-day precision (CV %), accuracy (bias %) and recovery data for the measurement of levofloxacin in human plasma (the analysis of five different samples, n = 5)

Nominal concentration	Found concen-tration mean		CV	Bias	Recovery	
(μg/mL)	μg/mL	± SD	(%)	(%)	(%)	± SD
0.100	0.103	0.010	9.3	3.2	95.5	4.1
0.200	0.209	0.006	3.1	4.6	97.6	5.8
0.800	0.779	0.050	6.5	-2.6	97.2	3.2
3.200	3.216	0.102	3.2	0.5	95.2	8.6

Table 3. The inter-day precision (CV %), accuracy (bias %) and recovery data for the measurement of levofloxacin in human plasma (one analysis on five different days, n = 5)

Nominal concentration (μg/mL)	Found concentration mean μg/mL	± SD	CV (%)	Bias (%)	Recovery (%)	± SD
0.100	0.104	0.011	11.0	3.9	95.9	4.2
0.200	0.203	0.019	9.5	1.5	97.3	3.7
0.800	0.762	0.044	5.8	-4.7	104.5	5.0
3.200	3.133	0.209	6.7	-2.1	98.5	1.3

CONCLUSION

The developed and validated LC-MS/MS assay is simple, rapid, and accurate having the characteristics required of the methods applied in therapeutic drug monitoring. The method was validated over the concentration range of 0.100-10 μg/mL which covers therapeutic plasma levels of levofloxacin. In comparison with other published HPLC [5, 14-20] or LC-MS/MS [21-23] methods for monitoring levofloxacin in human plasma, the developed method performs better in terms of volume of analyzed plasma sample, analyte recovery, and speed (both sample preparation and chromatographic run-time), which are essential attributes for methods used in routine analysis. This new fast method was successfully applied in therapeutic drug monitoring of levofloxacin. It can also be successfully used in pharmacokinetics and bioequivalence studies of levofloxacin.

EXPERIMENTAL SECTION

Reagents

Acetonitrile and methanol of isocratic grade for liquid chromatography, and formic acid of analytical-reagent grade were purchased from Merck KGaA (Darmstadt, Germany). Deionised water was obtained using a Milli-Q Water purification system (Millipore, Milford, MA, USA). The human blank plasma was supplied by the Regional Blood Transfusion Centre of Cluj-Napoca (Romania) from healthy volunteers, men and women.

Apparatus

The following apparatus were used: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical Plus and Precision Standard Balance (Mettler-Toledo, Switzerland); Vortex Genie 2 mixer (Scientific Industries, New York,

USA); Ultrasonic bath Elma Transsonic 700/H (Singen, Germany). The HPLC system used was an 1100 series Agilent Technologies model (Darmstadt, Germany) consisting of two G1312A binary pumps, an in-line G1379A degasser, an G1329A autosampler, a G1316A column oven and an Agilent Ion Trap Detector 1100 VL.

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a Zorbax SB-C18 (100 mm x 3.0 mm i.d., 3.5 μm) column (Agilent Technologies) under isocratic conditions using a mobile phase of a 17:83 (v/v) mixture of acetonitrile and 0.1% (v/v) formic acid in water at 50 ºC with a flow rate of 1 mL/min. The detection of levofloxacin was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer with an electrospray ion (ESI) source, positive ionisation (capillary 4000 V, nebulizer 60 psi (nitrogen), dry gas nitrogen at 12 L/min, dry gas temperature 350ºC). The extracted ion current (EIC) chromatogram of m/z 318 from m/z 362 was analysed.

Standard solutions

A stock solution of levofloxacin (5 mg/mL) was prepared by dissolving an appropriate quantity of levofloxacin in methanol. A working solution (10 μg/mL) was prepared by appropriate dilution in drug-free human plasma. This solution was used to prepare plasma calibration standards with the concentrations of 0.100, 0.200, 0.400, 0.800, 1.600, 3.200, and 10.00 μg/mL. Quality control (QC) samples of 0.200 μg/mL (low), 0.800 μg/mL (medium) and 3.200 μg/mL (high) were prepared by adding the appropriate volumes of working solution to drug-free human plasma. The resultant plasma calibration standards and quality control standards were pipetted into 15 mL polypropylene tubes and stored -20ºC until analysis.

Sample preparation

Standards and plasma samples (0.1 mL) were deproteinised with methanol (0.3 mL). After shaking with vortex-mixer (10 s) and centrifugation (5 min at 10.000 rpm), the supernatants (0.2 mL) were transferred in autosampler vials and 1 μL were injected into the HPLC system.

Method validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the plasma samples containing levofloxacin with those obtained from different plasma blank samples (n=6).

The concentration of levofloxacin was determined automatically by the instrument data system using peak areas and the external standard method. The calibration curve model was determined by the least squares analysis: $y = b + ax$, weighted ($1/y^2$) linear regression, where y - peak area of the analyte and x - concentration of the analyte (µg/mL).

The intra-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %) were determined by the analysis of five different samples (n = 5) from each QC standards (at lower, medium and higher levels) on the same day. The inter-day precision and accuracy were determined by analysis on five different days (n = 5) of one sample from each QC standards (at low, medium and high levels).

The lower limit of quantification (LLOQ) was established as the lowest calibration standard with an accuracy and precision less than 20%.

The relative recoveries (at LLOQ, low, medium and high levels) were measured by comparing the response of the spiked plasma with the response of standards in solvent with the same concentration of levofloxacin as the plasma (n = 5).

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