A simple and rapid high performance liquid chromatography method to determine levofloxacin in human plasma and its use in a bioequivalence study

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ABSTRACT: A simple and rapid HPLC-UV method has been developed for determination of levofloxacin in human plasma. Chromatographic separation was performed on a Kromasil C_{18} column with the mobile phase consisting of acetonitrile, water, phosphoric acid, and triethylamine (14:86:0.6:0.3, v/v/v/v) and flow rate was 1.0 mL/min. The method used ultraviolet detection set at a wavelength of 294 nm. The standard curves were linear over a concentration range of 0.05-5.0 μg/mL (r > 0.99). The method was simple, rapid, precise, accurate, and suitable for routine bioequivalence study. The method was successfully used in a bioequivalence study of two different levofloxacin hydrochloride capsules in healthy Chinese volunteers.

Key Words: Levofloxacin, high performance liquid chromatography, bioequivalence

1. Introduction

Levofloxacin, one of the commonly used fluoroquinolone antimicrobials, is the active S-isomer isolated from the racemic ofloxacin. Its antibacterial action is twice as active as the racemate ofloxacin *in vitro*. Levofloxacin possesses a broad spectrum of activity against various bacteria, including gram-positive and gram-negative microorganisms (*1*). It is also active against causes of atypical respiratory infection such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* (*2*). Because of its excellent antibacterial activity and low frequency of adverse effects on oral administration, levofloxacin has been widely used for the treatment of infectious diseases, such as community-acquired pneumonia and acute exacerbation of chronic bronchitis (*3*).

Numerous HPLC methods for the quantification of levofloxacin in biological samples have been reported. These methods involved the use of protein precipitation (*4*) and liquid-liquid extraction (*5*,*6*) and full automated extraction (*7*) and solid-phase extraction (*8*) coupled with ultraviolet detection and protein precipitation (*9*) and solid-phase extraction (*10*) followed by fluorescence detection. Capillary electrophoresis and nuclear magnetic resonance spectroscopy have also been used to determine drugs in biological fluids (*11*,*12*). The high performance liquid chromatography-tandem mass spectrometry method (HPLC/MS/MS) has been used to determine levofloxacin in human plasma (*13*). Recently, use of the hydrophilic interaction liquid chromatography-tandem mass spectrometry method (HILIC/MS/MS) to quantify levofloxacin has also been described (*14*). However, these methods suffer from a number of disadvantages, including tedious and time-consuming sample preparation and insufficient selectivity and stability.

In bioequivalence studies, the proposed method should be simple and able to process hundreds of samples in a limited time. This paper describes a simple, rapid, precise, and accurate HPLC method for determining levofloxacin in human plasma. Once developed and validated, this method was successfully used for bioequivalence investigation of two different levofloxacin hydrochloride capsules in 20 healthy Chinese volunteers.

2. Materials and Methods

2.1 *Chemicals and reagents*

Levofloxacin (99.7% purity) and ciprofloxacin (99.5% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (HPLC-grade) was obtained from Concord Tech (Tianjin, China) and the other chemicals and reagents were of analytical grade. Drug-free human plasma was provided by the central

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blood bank of Shenyang (Liaoning, China).

2.2 *Chromatographic conditions*

The HPLC system (JASCO, Kyoto, Japan) used consisted of a model PU-2080 pump, a fixed injection-loop of 20 μL, and a model UV-2075 UV detector; data acquisition was performed with the Sepu3000's processor (Hang Zhou, China). The analytical column employed was a Kromasil C_{18} column (200 mm × 4.6 mm, i.d., 5 μm) with a guard column (10 mm × 4.6 mm i.d., 5 μm) of the same packing material. The mobile phase consisted of acetonitrile, water, phosphoric acid, and triethylamine (14:86:0.6:0.3, v/v/v/v) and was filtered through a 0.45 μm cellulose membrane filter (Tianjin Auto Science, China) and degassed before use. The detection wavelength was set at 294 nm. Chromatography separation was performed at room temperature and flow rate was maintained at 1 mL/min.

2.3 *Standard solutions and quality control samples*

Primary stock solutions of levofloxacin (1 mg/mL) and ciprofloxacin (1 mg/mL) were prepared in water. Levofloxacin stock solution was further diluted with water to obtain different working standard solutions ranging from 0.25 to 25.0 μg/mL. A working standard solution of ciprofloxacin was prepared by diluting the stock solution with water to yield a final concentration of 25.0 μg/mL. Human plasma calibration standards of levofloxacin (0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 μg/mL) were prepared by spiking an appropriate amount of the working standard solutions into drug-free human plasma. Quality control (QC) samples were prepared at low (0.1 μg/mL), medium (0.5 μg/mL), and high (4.0 μg/mL) concentrations.

2.4 *Sample preparation*

To a 5-mL test tube were added 0.5 mL plasma, 100 μL of the internal standard working solution (25.0 μg/mL), and 100 μL of water. Then, 100 μL of perchloric acid (0.6 M) were added to precipitate protein in plasma; the result was vortex-mixed for 30 sec and centrifuged at 10,000 × g for 5 min, and a 20-μL aliquot of the supernatant was injected into the HPLC system.

2.5 *Validation of this method*

Six randomly selected blank plasma samples were processed by a similar extraction procedure and analyzed to determine the extent to which endogenous plasma components may contribute to interference at the retention time of levofloxacin and ciprofloxacin. The calibration curves were constructed each day before the analysis of the samples by plotting the peak-area ratio (levofloxacin/ciprofloxacin) versus the drug concentrations. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a coefficient of variation (CV) of less than 20% and accuracy of 80-120%. The accuracy and precision of the method were assessed by determining QC plasma samples at concentrations of 0.1, 0.5, and 4.0 μg/mL on three consecutive days, accompanying by a standard calibration curve on each analytical run. The recovery of levofloxacin was evaluated by comparing peak areas of pre-treated quality control plasma samples (n = 6) with mean peak areas of those spiked-after-extraction samples at the same nominal concentrations. The recovery of I.S. from plasma was evaluated at a concentration of 5.0 μg/mL using the same process. Stability quality control plasma samples were subjected to short-term (24 h) incubation at room temperature, three freeze/thaw cycles, and storage for 30 days (-20ºC).

2.6 *Bioequivalence study*

The present method has been used in a randomized crossover bioequivalence study in which the concentration of levofloxacin was measured in plasma samples from twenty healthy male Chinese volunteers after single oral doses (2 × 100 mg) of two different levofloxacin hydrochloride capsules from either YangZiJiang (reference, JiangSu , China) or DiSha (test, WeiHai, China) pharmaceutical companies under fasting conditions. After a one-week wash-out period, the subjects were crossed-over. Blood samples (3 mL) were drawn from the forearm at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 11, 15 and 24 h after administration, transferred to heparinized tubes, and gently mixed. After centrifugation (3,000 × g, 10 min), the separated plasma samples were collected and stored at -20ºC prior to analysis. Pharmacokinetic parameters were calculated by DAS Software-Version 2.0 (Chinese Pharmacological Society, China).

3. Results

3.1 *Specificity*

The current method showed excellent chromatographic specificity with no endogenous plasma interference at the retention times of levofloxacin and ciprofloxacin. Chromatograms obtained from human blank plasma and human blank plasma spiked with levofloxacin (2 μg/mL) and ciprofloxacin (5 μg/mL) are shown in Figure 1A and B, respectively. Levofloxacin and ciprofloxacin were well resolved with respective retention times of 8.4 and 9.4 min. Figure 1C shows the chromatogram of a plasma sample obtained at 1.0 h after a single oral dose of 200 mg levofloxacin from a healthy volunteer.

3.2 *Calibration curve and limit of quantification*

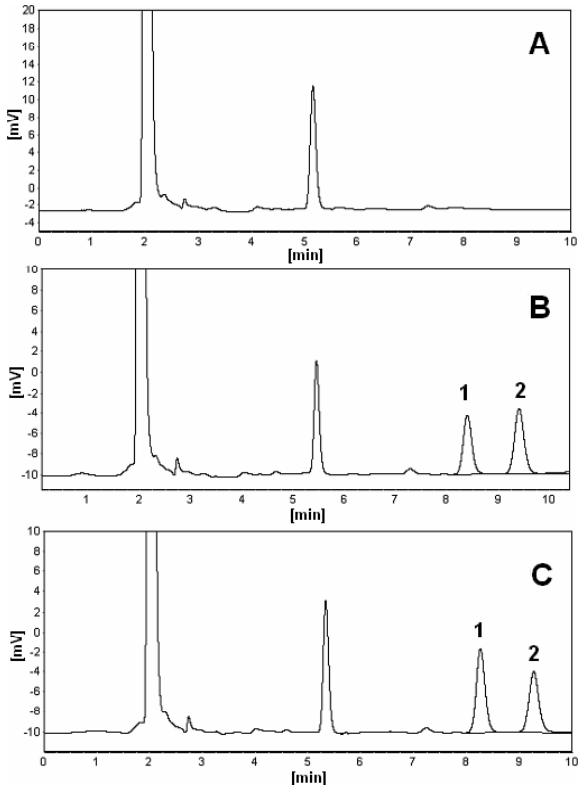

Figure 1. Representative chromatogram of human blank plasma (A), human blank plasma spiked with levofloxacin (1) and (2) (B), and plasma sample obtained at 1.0 h after a single oral dose of 200 mg of levofloxacin from a healthy volunteer (C).

Table 1. Accuracy and precision from the determination of levofloxacin in human plasma (n = 18)

Concentration (μg/mL)	Mean ± SD (μg/mL)	RE (%)	RSD (%)	
			Intra-day	Inter-day
0.1	0.098 ± 0.008	-1.9	12.4	6.9
0.5	0.477 ± 0.023	-4.7	6.0	4.4
4.0	4.27 ± 0.16	6.9	7.3	2.9

Table 2. Stability data of levofloxacin in human plasma

Concentration	Mean ± SD (μg/mL)	RE (%) (μg/mL)	RSD (%)
Short-term stability for 24 h in plasma at room temperature			
0.1	0.105 ± 0.008	4.6	7.5
0.5	0.527 ± 0.009	5.4	1.6
4.0	3.99 ± 0.093	-0.3	2.3
Long-term storage at -20ºC for 30 days			
0.1	0.095 ± 0.005	-4.7	5.0
0.5	0.495 ± 0.029	-0.9	5.8
4.0	4.15 ± 0.262	3.9	6.3
Three freeze/thaw cycles			
0.1	0.092 ± 0.005	-8.2	5.7
0.5	0.490 ± 0.013	-2.0	2.6
4.0	4.15 ± 0.041	3.9	1.0

The calibration curves were linear over the concentration range of 0.05-5.0 μg/mL with a mean correlation coefficient of 0.9972. The mean (± SD) regression equation from replicate calibration curves on different days was: Y = (0.4677 ± 0.0294)X + (0.0045 ± 0.0083), where Y is the peak area ratio of levofloxacin to ciprofloxacin and X is the plasma concentration of levofloxacin. The lower limit of quantification with a coefficient of variation of less than 20% was 50 ng/mL.

3.3 *Precision and accuracy*

The coefficient variation values of both inter- and intra-day analysis were less than 12.4% whereas the relative error was less than 6.9%. The inter- and intra-day precision and accuracy values of the assay method are presented in Table 1.

3.4 *Recovery*

The mean extraction recoveries of levofloxacin at concentrations of 0.1, 0.5, and 4.0 μg/mL were 86.9 ± 6.1, 95.0 ± 4.9 and 93.3 ± 3.7%, respectively. The extraction recovery of the I.S. was 93.5 ± 4.5%.

3.5 *Stability*

Treated plasma samples were found to be stable at least 24 h when the samples were kept at room temperature (RE < 5.4%). The concentrations of levofloxacin in plasma which underwent three freeze-thaw cycles or storage at -20ºC for 30 days were found to be stable with relative error of less than 8.2%. The stability data of levofloxacin stored under various conditions and subjected to freeze-thaw cycles are shown in Table 2.

4. Discussion

4.1 *Preparation of plasma samples*

Protein precipitation has the advantages of simplicity and universality, so PPT was used to prepare the plasma samples.

Protein precipitation with methanol, acetonitrile, and perchloric acid has been investigated, but when a supernatant treated with methanol or acetonitrile was injected into HPLC system a poor peak was obtained. Direct protein precipitation with perchloric acid was simple and rapid and good separation of the drug and I.S. was achieved using the precipitation method. Results indicated that 0.6 M perchloric acid per 100 μL plasma successfully removed all of the protein in the plasma. The use of a lower amount of perchloric acid results in incomplete precipitation, but a higher amount may reduce chromatographic column lifetime. Thus, 0.6 M perchloric acid was selected as the protein precipitation

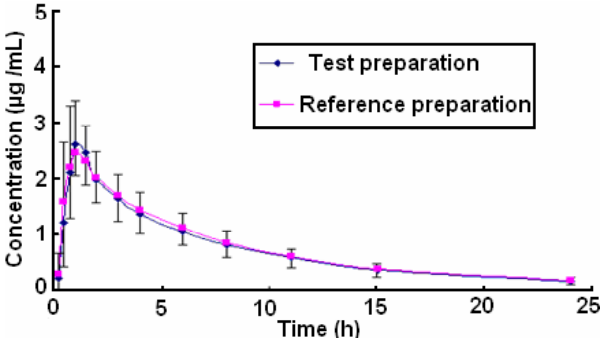

Figure 2. Mean plasma concentration-time curve of levofloxacin in a test with oral administration of a 200 mg levofloxacin hydrochloride or reference preparations to 20 healthy volunteers.

Table 3. Main pharmacokinetics parameters of levofloxacin (Mean ± SD, n = 20)

Pharmacokinetic parameters	Test preparation	Reference preparation
$AUC_{0\text{-}24}$ (μg·h/mL)	17.12 ± 3.83	17.55 ± 3.36
$AUC_{0\text{-}\infty}$ (μg·h/mL)	19.27 ± 4.15	19.26 ± 3.18
C_{max} (μg/mL)	2.95 ± 0.54	3.03 ± 0.87
T_{max} (h)	1.1 ± 0.3	1.1 ± 0.4
$t_{1/2}$ (h)	7.21 ± 3.01	7.69 ± 1.34
K_e (h^{-1})	0.09 ± 0.01	0.10 ± 0.01

agent.

4.2 *Optimization of mobile phase*

The chromatographic conditions were optimized by injecting analytes with mobile phase containing varying percentages of organic phase to achieve good resolution and symmetric peak shapes for levofloxacin and ciprofloxacin, as well as a short retention time. As expected, the retention times and resolutions of levofloxacin and the I.S. increased with decreasing acetonitrile percentage. The high organic solvent content shortened the chromatographic cycle time and an acidic modifier (phosphoric acid) in the mobile phase ensured stable chromatographic retention times for levofloxacin and ciprofloxacin. Triethylamine was added to improve peak shapes. Thus, optimal conditions were a mobile phase consisting of acetonitrile, water, phosphoric acid, and triethylamine (14:86:0.6:0.3, v/v/v/v). Under optimum conditions, the chromatographic run time for each sample was completed within 10 min.

4.3 *Advantages of the method*

In comparison to previously published HPLC methods for separation and quantitation of levofloxacin, the major modifications incorporated into the current method include: simple sample preparation procedures, common and cheap HPLC equipment and mobile phase additives, and a short analysis time as well. Thus the assay is suitable for routine analysis of large batches of biological samples to adjust drug dosage or perform pharmacokinetic studies. A simple, rapid, precise, and accurate HPLC method for determining levofloxacin in human plasma has been presented. Although lower sensitivity was obtained in comparison to previously published LC methods with fluorescence or mass spectrometry detection, the resulting LOQ (50 ng/mL) was sufficient for human pharmacokinetic studies.

4.4 *Bioequivalence evaluation*

The mean plasma concentration-time profiles and mean pharmacokinetic parameters after a test with a single oral dose of 200 mg and reference products administrated to twenty healthy male subjects are presented in Figure 2 and Table 3, respectively. Both the mean values and SD for the two preparations were close together. Pharmacokinetic parameters of the two preparations obtained from the statistical calculation exhibited bioequivalence.

5. Conclusion

An analytical method developed for levofloxacin quantification in plasma samples showed good specificity, sensitivity, linearity, precision, and accuracy over the entire range of clinically significant and therapeutically achievable plasma concentrations, thereby enabling its use in bioequivalence trials. Results of such a trial with the method revealed that test levofloxacin hydrochloride capsules from DiSha (WeiHai, China) and reference levofloxacin hydrochloride capsules from YangZiJiang (JiangSu, China) are bioequivalent.

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