Simple Extraction and Determination of Ofloxacin in Human Plasma by High – Performance Liquid Chromatography with Fluorescence Detector

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ABSTRACT

A specific, selective, sensitive and precise reversed-phase high-performance liquid chromatographic method has been developed for the determination of ofloxacin in human plasma. Pipemidic acid was used as an internal standard. The simple extraction method using protein precipitation with acetonitrile was employed for sample preparation. Good chromatographic separation was achieved by using ODS hypersil (5 µm, 250 x 4.0 mm) column and a mobile phase consisting of Acetonitrile : 25 mM Phosphoric acid + 2.5 mM N-Cetyl-N,N,N-trimethylammonium bromide (CTAB) pH=7.0 (20: 80) at a flow rate of 1.2 ml/min. Ofloxacin and pipemidic acid were detected with fluorescence detector at Ex = 285 nm, Em = 460 nm. No endogenous substances were found to interfere. Linearity range for ofloxacin was 25–4000 ng/ml. The coefficients of variation (%CV) for intraday and interday precision were less than 4.9 and 3.7% respectively, at all concentration levels while the intraday and interday accuracy ranged from 93.8–101.2 % at all concentration levels. This analysis method was successfully used in pharmacokinetic and bioequivalence study of ofloxacin in healthy volunteers.

Key words: Ofloxacin, Plasma analysis, HPLC, Determination, Pharmacokinetic

INTRODUCTION

Ofloxacin, a synthetic fluoroquinolone anti-infective agent has an expanded spectrum of activity and increased antibacterial potency compared with nonfluorinated quinolones (McEvoy, 2005).

Ofloxacin is used orally or IV in adults for the treatment of mild to moderate urinary tract infections, prostatitis, lower respiratory tract infection and skin structure infections caused by susceptible gram-negative and -positive aerobic bacteria. In addition, the drug is used in the treatment of acute, uncomplicated gonorrhea, disseminated gonococcal infections, nongonococcal urethritis and cervicitis caused by susceptible Chlamydia, mixed infection of urethra and cervix.
caused by *C. trachomatis* and *Neisseria gonorrhoeae*, and acute pelvic inflammatory disease (including severe infection) caused by *C. trachomatis* and/or *N. gonorrhoeae*.

Ofloxacin is rapidly and almost completely absorbed from the GI tract following oral administration. The drug does not undergo appreciable first-pass metabolism. Presence of food in the GI tract may decrease slightly the rate and/or extent of absorption of ofloxacin, however, this effect is not considered clinically important. The oral bioavailability of ofloxacin is 85-100% in healthy, fasting adult, and peak serum concentrations of the drug generally are attained within 0.5-2 hours. In healthy adults with normal renal function, the elimination half life of ofloxacin in the distribution phase averages 0.5-0.6 hour and the elimination half life in the terminal phase averages 4-8 hours.

Ofloxacin occurs as an off-white to pale-yellow, crystalline powder. At room temperature, ofloxacin has aqueous solubilities of 3.5-4 mg/ml at pH 7, 60 mg/ml at pH 2-5 and 303 mg/ml at pH 9.8. The pKas of the drug are 5.74 and 7.9 (McEvoy, 2005). The chemical structures of ofloxacin and pipemidic acid are presented in Figure 1.

![Chemical structures of ofloxacin and pipemidic acid](image)

**Figure 1.** Chemical structures of ofloxacin and pipemidic acid as internal standard.

High – performance liquid chromatography has been developed for the determination of ofloxacin in biological fluid by fluorescence detector (Immanuel and Hemanth Kumar, 2001) which ofloxacin in human plasma was extracted by deproteinization with 7% perchloric acid. Another method using protein precipitation with acetonitrile followed by evaporation and enoxacin was used as an internal standard (Macek and Ptacek, 1995).

The purpose of the present study was to develop a simple HPLC analysis method for ofloxacin in human plasma determination with high specificity / selectivity, sensitivity, accuracy, precision and reproducibility.
MATERIALS AND METHODS

Chemicals and reagents Ofloxacin reference standard was obtained from Zhejiang East-Asia Pharmaceutical Chemical Co., Ltd. (China). Pipemidic acid was supplied by Sigma (USA). Analytical grade ortho-phosphoric acid 85% (Merck, Darmstadt, Germany), analytical grade N-Cetyl-N,N,N-trimethylammonium bromide (CTAB) (Merck, Darmstadt, Germany), analytical grade hydrochloric acid 37% (Merck, Darmstadt, Germany), isocratic grade acetonitrile (Merck, Darmstadt, Germany). Deionized water from a milli Q apparatus was used in this study. Plasma was purchased from a local blood bank to generate a drug-free plasma pool.

Apparatus and chromatographic conditions The HPLC system consisted of a model LC-10ATvp pump (Shimadzu, Kyoto, Japan), a model DGU-14A degasser (Shimadzu, Kyoto, Japan) and a model SIL–10ADvp auto injector (Shimadzu, Kyoto, Japan). Separation was achieved on ODS hypersil (5 μm) cartridge column (250 x 4.0 mm I.D) (Agilent Tecnologies, U.S.A) and detected with fluorescence detector model RF-10Axl (Shimadzu, Kyoto, Japan) at Ex = 285 nm, Em = 460 nm.

The part of extraction consisted of a model 2601 multi–tube vortexer (Scientific Manufacturing Industries, U.S.A), abbott centrifuge (Abbott Laboratories, Germany).

The mobile phase consisted of acetonitrile - 25 mM phosphoric acid + 2.5 mM CTAB pH = 7.0 (20: 80). It was filtered with 0.25 μm membrane filter (Sartorius, Germany) before use. Chromatography was performed at ambient temperature. Flow rate was 1.2 ml/min and injection volume was 50 μl.

Stock and standard solution Stock solutions of ofloxacin were prepared by dissolving 25 mg in 25 ml 0.1 N hydrochloric acid to yield a final concentration of 1 mg/ml and stored at 4°C until analysis. This stock solution was used to prepare a set of working standard by diluting in deionized water. A 25 μl of each concentration of working standard was pipetted into 225 μl of drug-free human plasma to yield concentrations of 25, 50, 100, 500, 1000, 2000 and 4000 ng/ml. In the same manner, stock solution of ofloxacin QC sample was separately prepared at concentration of 1 mg/ml which using for plasma quality controls (QC) preparation. Three concentrations of QC sample (75, 750 and 3000 ng/ml) were prepared in plasma and included in every analytical run. Internal standard solution (pipemidic acid) was prepared in .1 N hydrochloric acid and working internal standard was diluted with acetonitrile. A 1000 μl of internal standard working solution (7.5 μg/ml) was added to a 250 μl of drug-free human plasma to yield a concentration of 6 μg/ml. Plasma standards were extracted by the process mentioned above before injection into the HPLC system.

Analytical procedure 250 μl aliquot of the spiked plasma or human plasma sample was pipetted into a microcentrifuge-tube and 1000 μl of 7.5 μg/ml pipemidic acid in acetonitrile (internal standard) was mixed. The sample tubes were closed with a cap. After that the contents were vortex-mixed for 15 minutes and centrifuged at 10900 rpm for 15 minutes. The supernatant was decanted to a clean test tube (16 x 100 mm, Kimax®, USA) and then diluted by using
1500 μl of 25 mM Phosphoric acid + 2.5 mM CTAB pH=7.0. Then supernatant was collected into a clean vial and 50 μl was injected onto the HPLC column.

Validation of the analysis method including specificity/selectivity, extraction recovery, accuracy and precision, sensitivity and stability of the sample was performed accordingly to the Guidance for Industry: Bioanalytical Method Validation (Guidance for Industry, 2001).

**Specificity and selectivity** Peaks of drug and internal standard were separated from other interfering peaks in blank plasma by comparing the chromatograms of the following samples:
1. blank plasma of 6 normal volunteers
2. standard solution of ofloxacin and the internal standard (pipemidic acid)
3. blank plasma spiked with ofloxacin and the internal standard

**Recovery (extraction efficiency)** Extraction recoveries of ofloxacin and pipemidic acid from human plasma were determined by comparison of HPLC responses (peak area) from extracted sample (quality control sample), containing known amounts (75, 750, 3000 ng/ml for ofloxacin and 6 μg/ml for pipemidic acid), to those from unextracted and directly- injected standard, spiked with the same amount.

**Accuracy and Precision** Accuracy and precision of the method were determined by five replicate analysis of known ofloxacin concentration over the calibration curve. Interday (between – run) and intraday (within-run) accuracy were expressed as percentage from spiked concentration, using following equation:

\[
\% \text{ Accuracy} = \left( \frac{C_{obs}}{C_{spike}} \right) \times 100
\]

Where \( C_{obs} \) is the observed concentration for each standard, and \( C_{spike} \) is the spiked theoretical concentration. Intraday (within-run) precision was studied by analyzing 5 sets of plasma spiked with ofloxacin on the same day. Interday (between – run) precision was determined by quantifying the observed concentrations of quality control (QC) sample at three concentration levels, i.e., 75 ng/ml (low conc.), 750 ng/ml (medium conc.), and 3000 ng/ml (high conc.) on 5 different days along with a daily – prepared standard curve. Interday and intraday precision of the method was expressed as the coefficient of variation (C.V.) of the mean peak ratio for each standard or QC sample.

**Calibration curve** Calibration curves were constructed in the range of 25 – 4000 ng/ml . The curves were obtained daily for 5 days by plotting between the peak area ratio and concentrations of standards.

**Stability** To determine the influence of different times on the stability of drug, ofloxacin was spiked to blank plasma at the concentrations of 75, 750 and 3000 ng/ml, each portion of spiked plasma was repeatedly analyzed five times for ofloxacin concentration (n=5) under different storage conditions at – 40°C. The first portion (initial concentration) was immediately extracted and analyzed as mentioned above, another portion was extracted and analyzed at time described under the following stability studies. The stability of ofloxacin was expressed as
observed concentration of ofloxacin comparing with initial concentration (t=0). This study will be referred to in detail as follows:

Freeze-thaw stability and long-term stability The study of freeze-thaw stability and long term stability were performed by analyzing the spiked plasma samples after 3 cycles of freeze-and-thaw and after being stored for 14 days, respectively.

Bench-top stability The study of bench top stability was performed by analyzing the spiked plasma samples after sitting on a laboratory bench at room temperature for 5 hours.

Autosampler stability The study of autosampler stability was performed by analyzing the spiked plasma samples immediately and then repeated after 24 hours.

RESULTS AND DISCUSSION

The simple extraction method, utilizing protein precipitation with acetonitrile, was used for sample preparation. Good chromatographic separation was achieved by ODS hypersil (5 μm, 250 x 4.0 mm I.D) column, and mobile phase consisting of acetonitrile - 25 mM phosphoric acid + 2.5 mM CTAB pH=7.0 (20:80) at flow rate of 1.2 ml/min. The optimum wavelength was detected with fluorescence detector at Ex = 285 nm, Em = 460 nm which had much better detector response.

No interfering peak was observed at the retention times of both ofloxacin and the internal standard. The retention times of either ofloxacin or the internal standard were approximately the same in all chromatograms (Figure 2).

The method was validated with regard to linearity, limit of detection and qualification, recovery, precision, accuracy and specificity.

The lowest standard on the calibration curve should be accepted as the lower limit of quantification (LLOQ) when the response at LLOQ is at least 5 times greater than that of blank response and the analyte peak (response) should be identifiable, discrete and reproducible with high precision (% CV ≤ 20 and accuracy of 80 – 120%). The lowest concentration on the standard curve with acceptable accuracy, precision and variability was 25 ng/ml with the coefficient of variation (CV) of 3.0% and the percent accuracy of observed plasma ofloxacin concentration (n=5) from the spiked concentration was 109.8%. Therefore, this concentration was accepted as the lower limit of quantification.
Figure 2. Representative chromatograms of blank plasma from a volunteer (A), Plasma standard containing 500 ng/ml ofloxacin (peak II) and internal standard (peak I). (B), Plasma standard containing 25 ng/ml Ofloxacin (peak II) and internal standard (peak I) (C).

Peak area ratio of ofloxacin of calibration standards were proportional to the plasma concentration of ofloxacin in the range of 25-4000 ng/ml. Each concentration was tested 5 times. The coefficient of correlation ($r^2$), slope and intercept to linear regression line are reported in Table 1.

Table 1. Statistical data (n=5) for linearity of ofloxacin.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$r^2$</th>
<th>slope (mean ± S.D)</th>
<th>intercept (mean ± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ofloxacin</td>
<td>0.9999±0.0001</td>
<td>0.0021±0.0002</td>
<td>-0.0032±0.0084</td>
</tr>
</tbody>
</table>

The absolute recovery was calculated by comparing the areas under the peak obtained from standard working solutions with the peak areas from standard sample. The recovery of ofloxacin (75, 750, and 3000 ng/ml) and pipemidic acid were 87.8±1.1%, 83.7±1.6%, 75.7±1.4% and 75.5±1.5%, respectively. The results
are shown in Table 2.

**Table 2.** Recovery of extraction for the analysis of ofloxacin and pipemidic acid in human plasma (n = 5).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentrations add (ng/ml)</th>
<th>% Recovery ± (S.D.)</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>ofloxacin</td>
<td>75</td>
<td>87.8±1.1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>83.7±1.6</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>75.7±1.4</td>
<td>1.9</td>
</tr>
<tr>
<td>pipemidic acid</td>
<td>6 (μg/ml)</td>
<td>75.5±1.5</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Intraday assay precision of the method is illustrated in Table 3. It was estimated by assaying the quality control samples five times in the same analytical runs. The precision was less than 4.8% and accuracy ranged from 95.5 – 100.3% at all concentration levels.

Interday assay precision and accuracy was evaluated by processing a set of calibration curves and quality control samples (three levels analyzed five times, results averaged for statistical evaluation) on five different days (Table 3). The precision was less than 3.7% and accuracy ranged from 93.8-101.2% at all concentration levels.

**Table 3.** Precision and accuracy of the HPLC method for the analysis of ofloxacin in human plasma.

<table>
<thead>
<tr>
<th>Conc. Add (ng/ml)</th>
<th>Intraday studies (n=5)</th>
<th>Interday studies (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. Found mean ± S.D(ng/ml)</td>
<td>%CV</td>
</tr>
<tr>
<td>75</td>
<td>71.6±1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>750</td>
<td>752.6±17.9</td>
<td>2.4</td>
</tr>
<tr>
<td>3000</td>
<td>3010.0±144.6</td>
<td>4.8</td>
</tr>
</tbody>
</table>

The stability of ofloxacin in plasma was determined by periodic analysis of spiked samples. The results indicated less degradation which suggested that ofloxacin had a good stability in human plasma, either short term or long-term stability test (Table 4).
Table 4. Stability of ofloxacin in human plasma (n = 5).

<table>
<thead>
<tr>
<th>Type of stability</th>
<th>Conc. (ng/ml) at t = 0, (mean ± S.D)</th>
<th>Conc. (ng/ml) at t = t, (mean ± S.D)</th>
<th>% Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze - thaw (3 cycle)</td>
<td>72.2±0.9</td>
<td>67.9±1.1</td>
<td>-5.9</td>
</tr>
<tr>
<td></td>
<td>757.6±10.7</td>
<td>682.6±6.7</td>
<td>-9.9</td>
</tr>
<tr>
<td></td>
<td>3011.9±65.3</td>
<td>2941.4±52.5</td>
<td>-2.3</td>
</tr>
<tr>
<td>Long - term (7 day)</td>
<td>72.2±0.9</td>
<td>73.6±2.0</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>757.6±10.7</td>
<td>755.1±28.7</td>
<td>-0.3</td>
</tr>
<tr>
<td></td>
<td>3011.9±65.3</td>
<td>3022.9±44.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Short - term (5 hour)</td>
<td>72.2±0.9</td>
<td>69.1±3.0</td>
<td>-4.3</td>
</tr>
<tr>
<td></td>
<td>757.6±10.7</td>
<td>752.0±25.6</td>
<td>-0.7</td>
</tr>
<tr>
<td></td>
<td>3011.9±65.3</td>
<td>2939.9±16.6</td>
<td>-2.4</td>
</tr>
<tr>
<td>Autosampler (24 hour)</td>
<td>72.2±0.9</td>
<td>67.1±0.4</td>
<td>-7.1</td>
</tr>
<tr>
<td></td>
<td>757.6±10.7</td>
<td>756.3±5.5</td>
<td>-0.2</td>
</tr>
<tr>
<td></td>
<td>3011.9±65.3</td>
<td>2916.3±89.5</td>
<td>-3.2</td>
</tr>
</tbody>
</table>

The simple extraction of ofloxacin in human plasma by protein precipitation with acetronitile without evaporation which produced satisfactory results in a shorter time than the method used by Macek and Ptacek (1995). Only 250 μl of plasma sample was used for sample preparation but still had high sensitivity (LLOQ = 25 ng/ml), which better than the method used by Immanuel and Hemanth Kumar (2001) but similar with that of Macek and Ptacek (1995). Thus, this developed analysis method was suggested to be more suitable for ofloxacin pharmacokinetic and bioavailability study.

The developed method was applied to determine plasma ofloxacin in pharmacokinetic and bioavailability study in 12 healthy male volunteers. Plasma samples were periodically collected up to 24 hours after oral administration of 300 mg ofloxacin tablet (Tarivid®). Figure 3 illustrates the mean ± SD plasma concentration time profile of ofloxacin, following an oral dose of 300 mg ofloxacin. The plasma level of ofloxacin reached the maximum in about 0.75 hour after administration and calculated half-life of elimination (t 1/2) of ofloxacin was 5.2 ± 1.7 hours, AUC0 --> inf was 24.0 ± 6.3 μg.hr/ml. The results are similar to those reported in AHFS Drugs Information (McEvoy, 2005).
Figure 3. Mean plasma concentration time profile of ofloxacin after oral administration of 300 mg ofloxacin tablet in 12 healthy male volunteers.

CONCLUSION

This developed HPLC method for the determination of plasma ofloxacin concentration was found to be a highly effective method since it used one-step precipitated extraction with acetonitrile without followed evaporation. It is also simple with low cost of extraction and high extraction recovery. Moreover, this analysis method had high specificity and selectivity, sensitivity, accuracy and precision and also had a good stability, either short-term or long-term. The analytical method was successfully used in pharmacokinetic and bioequivalence study of ofloxacin in healthy volunteers.

ACKNOWLEDGEMENTS

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REFERENCES
