A new approach of ofloxacin analysis method in human blood plasma using solid-phase extraction - high-performance liquid chromatography - ultra violet

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INTRODUCTION

Ofloxacin (OFX) is a second generation formula of quinolone broad spectrum antibiotics (Figure 1). OFX is widely used for infections of the eye, urinary tract, digestive tract, respiratory tract, skin and soft tissue, joints and bones, infections by pneumococcus resistant to beta-lactam antibiotics, and macrolides, as well as for treating diseases transmitted through intercourse. The working mechanism of OFX inhibits bacterial protein synthesis, which in turn arrests topoisomerase II enzyme with the enzyme DNA gyrase and IV.1

Previous studies have investigated OFX in the biological fluid matrix, such as by the method of protein deposition in high-performance liquid chromatography (HPLC) with ultra violet (UV) detector. The methods of solid-phase extraction (SPE) - HPLC with fluorescence detector,5 photodiode array detector,6 and liquid chromatography-mass spectrometry7 have been investigated. However, research analysis of OFX in human blood plasma using SPE for HPLC with UV detectors has not been reported. SPE extraction method is more effective compared to other extraction methods, one of the advantages being that it can isolate samples despite its small concentration in the matrix.7,8

Sample pretreatment is the main part that determines the efficacy in the analysis of drug or metabolites in a biological matrix. SPE is one solution since it can establish recovery and reproducibility of the matrix interference.9,11 The parent metabolite compound (such as carbohydrate, protein, and lipid) should be eliminated as the presence of interference can cause misleading results in assay methods.12 Therefore, it is necessary that the method can identify both the parent drug and metabolites accurately. The use of SPE in sample preparation can reduce time and solvent volume.13,14 In the previous research, application of SPE in the determination of lead compounds of

ABSTRACT

Objective: The aim of this research was to develop a method for analyzing ofloxacin (OFX) assay in human plasma using solid-phase extraction (SPE)- high-performance liquid chromatography (HPLC)/ultra violet (UV) detector. In this work, SPE was employed in preparing for the analysis of OFX using HPLC-UV detector. Materials and Methods: Hydrophilic and lipophilic balance cartridge (100 mg, particle size 10 µm) of SPE was used in preparing a sample to determine further method of analysis using HPLC with phosphate buffer 0.025 M (pH 2.5) and acetonitrile (85.5:14.5) as mobile phase and a flow rate of 1.2 ml/min. UV detector was adjusted at 295 nm using internal standard ciprofloxacin. Results: Calibration curve was linear over the range of 0.1-6 µg/ml with correlation coefficient (r) = 0.9998-0.9999. The resolution was (Rs) > 1.5, and repeatability (% CV) <10%. Based on peak area and the peak height ratio of chromatogram, limit of detection and limit of quantification were 0.023 µg/ml and 0.076 µg/ml, respectively, and recovery of spiked OFX in human plasma was 94.32-100.45%. Conclusion: Based on the results of analysis, the analysis method was concluded as sensitive and valid for analysis of OFX in human plasma.

KEY WORDS: High-performance liquid chromatography, Human plasma, Ofloxacin, Solid phase extraction

Access this article online

Website: jprsolutions.info ISSN: 0974-6943

Received on: 16-06-2017; Revised on: 28-07-2017; Accepted on: 13-08-2017

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aromatherapy in mice’s blood plasma after inhalation of essential oil yielded reliable (recovery 90%) and reproducible (CV <15%) results.

Based on the chemical structure, OFX contains a chromophore; consequently, its analysis in human blood plasma can be done using HPLC-UV detector with optimum results and good validity. We hope that the results of this work can be used as a reference in OFX bioavailability and bioequivalence studies, as validation of developing methods in the pharmaceutical industry and clinical laboratory, especially for routine analysis.

**MATERIALS AND METHODS**

**Materials**

OFX obtained from Zhejiang Jinxin, China, ciprofloxacin (CFX) purchased from Zhejiang Jinxin, China. Sodium dihydrogen phosphate monohydrate from Merck, Pro analytical grade, acetonitrile from Merck, HPLC grade and phosphoric acid from Merck, Pro analytical grade.

**Instruments**

HPLC (Shimadzu LC-10 ATVP), UV-VIS SPD Detector, auto injector Shimadzu System Controller SCL-A, column (Phenomenex); (250 mm, D 4.6 mm, particle size 10 µm), spectrophotometer UV-visible (analytical Jena, specord 200).

**Methods**

**Preparation of mobile phase**

A phosphate buffer 0.025 M (pH 2.3) and acetonitrile was mixed in ratio (85:15); (85.5:14.5) and (86:14) v/v. The mixture was filtered and vacuumed using Millipore 0.45 µm and an ultrasonic bath for 15 min.

**Preparation of Stock Solution and Determination of Maximum Wavelength**

The accurate weight of OFX amount 100 mg, dissolved in 200 ml measuring flasks with mobile phase up to the final concentration of 0.5 mg/ml, and it was diluted using mobile phase up to the final concentration of 5 µg/ml. A solution was scanned using UV-visible spectrophotometer in wavelength range 200-320 nm. The maximum absorbance of spectrum was observed to define the maximum wavelength of OFX. The same procedure was done for CFX.

**Determination of Molar Extinction**

Concentration of OFX solution with concentration 6.9 and 13.5 µM is measured at a maximum wavelength of OFX and calculated the value of the molar extinction base on its absorbance using Lambert-Beer equation.

**Optimization of HPLC Condition**

The solution of OFX 0.1 mg/ml containing (CFX) 0.1 mg/ml as internal standard (IS) was injected 10 µl (auto injectors) into the HPLC tool with several ratios of mobile phase composition (85:15), (85.5:14.5) and (86:14) v/v and flow rate 1.2 and 1.3 ml/min. A retention time and the resolution of the two peaks (CFX and OFX) were observed to determine the optimum condition. Optimization condition was selected for mobile phase composition, and flow rate accordingly showed a retention time is faster as possible, and resolution of two peaks results a value more than 1.5.

**Determination of SPE Extraction Recovery**

Into the SPE cartridge added 1 ml of methanol and Aqua bides with the assist of a vacuum (conditioning steps), added 1 ml of plasma was spiked with OFX concentration (0.10, 0.25, 1.00, 2.00, 3.00, 4.00 5.00, and 6.00, µg/ml), each concentration contained CFX 3 µg/ml (loading steps). Added 1 ml 5% methanol (washing steps), eluted analysts with 20% acetonitrile 1 ml in phosphate buffer (eluting steps). Analytics were injected into the HPLC at optimum condition, and then the efficiency of extraction of SPE was calculated by comparing the area under the curve (AUC) between chromatograms of SPE and without SPE treatments for same concentration.

**Validation of Analytical Method**

**Selectivity**

Selectivity determined by observing the results of the peak separation between CFX and CFX peak in chromatograms calculated the value of its resolution.

**Repeatability**

Repeatability is determined by making a solution of OFX 0.25 µg/ml in blood plasma and then extracted using the SPE. Analysts (10 µl) was injected into the optimum conditions HPLC; the experiment is repeated 6 times, then calculated coefficients of variation (% CV).

**Linearity**

Linearity was defined by preparing the calibration curves of five series OFX’s concentration (0.1, 0.25, 2, 4, and 6 µg/ml) and CFX 3 µg/ml as IS in blood plasma, and then each concentration was extracted using the SPE procedure. Each of analysts (10 µl) was injected into the optimum conditions of HPLC analysis; the experiment was repeated 3 times. The equation of a calibration curve was calculated using a least square regression model to obtain a correlation coefficient (r) and line regression equation. The equation which indicated the best value of (r) was used to determine the sample to the next steps.
Limit of Detection (LOD) and Limit of Quantification (LOQ)
The value of the LOD and LOQ was calculated statistically using a linear regression line of OFX calibration curve using formula below:

\[
\text{Residual standard deviation } \left( \frac{\text{Sy}}{\bar{x}} \right) = \sqrt{\frac{\sum (Y_i - \bar{Y})^2}{n-2}}
\]  

\[\text{LOD} = \frac{3\text{Sy}}{b}\]  

\[\text{LOQ} = \frac{10\text{Sy}}{b}\]

Accuracy and Precision
Accuracy and precision were employed by making a solution of OFX sample with final concentration 1, 3, and 5 µg/ml with CFX (3 µg/ml) as IS in blood plasma, subsequently each sample was extracted using SPE process. Amount 10 µl of analysts was injected into the optimum conditions HPLC. The experiment was repeated 3 times, and then the % recovery and precision (% CV) were calculated.

Suitability Studies of the HPLC System
The sample of OFX (0.25 µg/ml) and IS (3 µg/ml) in blood plasma was extracted using the SPE procedures. Afterward, it was injected (10 µl) into the optimum conditions of HPLC with six repetitions (n = 6). The % CV of the retention time, the AUC ratio and height peak ratio of chromatogram were calculated.

RESULTS

Determination of Wavelength OFX and CPX
The wavelength OFX and CPX have revealed the maximum absorption at a wavelength of 295 nm and 279 nm, respectively (Figure 2).

Molar Extinction of OFX
Molar extinction value has shown at Table 1, the average value was 33,238.89 M\(^{-1}\) cm\(^{-1}\).

Determination of SPE Extraction Recovery
The recovery extraction of SPE has exhibited value in the range 80-120%. Complete results of the recovery extraction available in Table 2.

Optimization of HPLC Condition
Optimization conditions of the HPLC analysis method conducted against parameters include chromatography retention time (Rt), resolution (Rs), the number of theoretical plates (N), the efficiency of the column (HETP) in various compositions, and the flow rate of mobile phase. HPLC condition optimization results can be seen in Table 3.

Validation of Analytical Method
Selectivity
The selectivity of analytical methods was represented by resolution (Rs). OFX’s peak Rt = 11.092 and CPX’s Rt = 12.533, value resolution (Rs) was 1.75 (Figure 3).
Table 1: Molar extinction ($\epsilon$) of OFX

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Molar concentration (M)</th>
<th>Absorbance</th>
<th>Molar extinction $\epsilon$ (M$^{-1}$cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0000069</td>
<td>0.223</td>
<td>32.333</td>
</tr>
<tr>
<td>2</td>
<td>0.0000135</td>
<td>0.473</td>
<td>35.066</td>
</tr>
<tr>
<td>3</td>
<td>0.0000180</td>
<td>0.582</td>
<td>32.316</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>99.716</td>
</tr>
<tr>
<td>$\bar{X}$</td>
<td></td>
<td></td>
<td>33.238</td>
</tr>
</tbody>
</table>

Table 2: Recovery extraction of OFX ($n$=3)

<table>
<thead>
<tr>
<th>Recovery of OFX (%)</th>
<th>Recovery of CFX (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Konsentrasi OFX ($\mu$g/ml)</td>
<td>In concentration of OFX ($\mu$g/ml)</td>
</tr>
<tr>
<td>0.25</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>109.37</td>
</tr>
<tr>
<td>2</td>
<td>107.18</td>
</tr>
<tr>
<td>3</td>
<td>108.49</td>
</tr>
<tr>
<td>$\bar{X}$</td>
<td>108.35</td>
</tr>
<tr>
<td>CV %</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Height peak ratio

<table>
<thead>
<tr>
<th>Recovery of OFX (%)</th>
<th>Recovery of CFX (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of OFX ($\mu$g/ml)</td>
<td>In concentration of OFX ($\mu$g/ml)</td>
</tr>
<tr>
<td>0.25</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>104.58</td>
</tr>
<tr>
<td>2</td>
<td>95.25</td>
</tr>
<tr>
<td>3</td>
<td>99.79</td>
</tr>
<tr>
<td>$\bar{X}$</td>
<td>100.21</td>
</tr>
<tr>
<td>CV %</td>
<td>4.17</td>
</tr>
</tbody>
</table>

Table 3: Composition optimization of mobile phase and flow rate

<table>
<thead>
<tr>
<th>Mobile phase composition: phosphate buffer (pH 2.2): Acetonitrile (%v/v)</th>
<th>Flow rate (ml/minute)</th>
<th>Rt OFX (minute)</th>
<th>Resolution ($R_s$)</th>
<th>Total theoretical plate (N)</th>
<th>HETP (L/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>86:14</td>
<td>1.2</td>
<td>14.250</td>
<td>1.88</td>
<td>3.4808</td>
<td>0.0718</td>
</tr>
<tr>
<td>85.5:14.5</td>
<td>1.3</td>
<td>12.750</td>
<td>1.83</td>
<td>3.3035</td>
<td>0.0757</td>
</tr>
<tr>
<td>5:15</td>
<td>1.3</td>
<td>11.375</td>
<td>1.77</td>
<td>3.3659</td>
<td>0.0743</td>
</tr>
</tbody>
</table>

Figure 3: Chromatogram of ofloxacin using ciprofloxacin as internal standard
Repeatability
A repeatability (% CV) based on retention time, chromatogram area ratio, and the height peak ratio of chromatogram were <10%. Test resulted of repeatability was served in Table 4.

Linearity
Linearity was obtained over ranging 0.1-6 µg/ml, a linear regression line equation based on the area ratio was used to determine OFX (y = 0.7687x + 0.0163; r = 0.999914). Based on the peak height ratio, and the equation of the linear regression line was y = 0.8638x + 0.0430 (r = 0.99990). The best equation of the calibration curve has shown in Figures 4 and 5.

LOD and LOQ
The value of LOD base on the AUC ratio and the peak height ratio of chromatogram were 0.0227 µg/ml and 0.0241 µg/ml, respectively. Values of LOQ according to the AUC ratio and the peak height ratio of chromatogram were 0.0757 µg/ml and 0.0804 µg/ml, respectively.

Accuracy and Precision
The value of accuracy showed the value 80-120% and the precision (% CV) was <10% (Tables 5 and 6).

System Suitability Test
A system suitability test has exhibited the value of % CV <2%. A system suitability test results were listed in Table 7.

DISCUSSION
The maximum wavelength of OFX is used in detection analysis with HPLC. Molar extinction of the OFX revealed values more than 10.000, indicating that it is possible to detect OFX by the UV detector in the HPLC system. This is due to the long chromophore system in OFX structure. Conditioning of SPE is carried out to clear exposure to pollutants on the SPE cartridge along storage and to maintain the moisture of the SPE cartridges. The value of recovery extraction fulfilled the requirement (>85%).[15]

The value of theoretically plate efficiency (N) shows the result of ≥2.500; it indicates that the peaks produced are sharp enough.[16] The mobile phase with a composition of 0.025 M phosphate buffer (pH 2.2) and acetonitrile with a ratio (85.5: 14.5) % v/v and flow rate 1.2 ml/min was chosen as the optimum condition for HPLC analysis, since the value resolution 1.77 (≥1.5) and retention time it produces has been relatively faster.[8]

Validation of Analytical Method
For the selected method represented by the resolution of peaks and the corresponding value of resolution (Rs) = 1.75, the requirements for value resolution are more than 1.5.[8,17] As the repeatability (% CV) of the area and peak height, chromatogram ratio was <2%, it may be said that the analysis samples of biological fluids fulfilled the requirement of <10%.[15,17] A linear regression line equation was approved through the correlation coefficient (r), and the value of r fulfilled the requirement of >(0.99).[8] LOD and LOQ absolute could be determined if the analyzed concentration was relatively small as in the biological matrix[18,19] the values of LOD and LOQ were sensitive enough.

Table 4: Repeatability of OFX

<table>
<thead>
<tr>
<th>Concentration of OFX (µg/ml)</th>
<th>Retention time (minute)</th>
<th>AUC ratio of chromatogram</th>
<th>Height peak of chromatogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>10.267</td>
<td>0.211</td>
<td>0.265</td>
</tr>
<tr>
<td></td>
<td>10.242</td>
<td>0.207</td>
<td>0.268</td>
</tr>
<tr>
<td></td>
<td>10.167</td>
<td>0.202</td>
<td>0.249</td>
</tr>
<tr>
<td></td>
<td>10.108</td>
<td>0.202</td>
<td>0.255</td>
</tr>
<tr>
<td></td>
<td>10.092</td>
<td>0.203</td>
<td>0.253</td>
</tr>
<tr>
<td></td>
<td>10.067</td>
<td>0.209</td>
<td>0.261</td>
</tr>
<tr>
<td>X</td>
<td>10.157</td>
<td>0.206</td>
<td>0.259</td>
</tr>
<tr>
<td>CV %</td>
<td>0.81</td>
<td>1.90</td>
<td>2.80</td>
</tr>
</tbody>
</table>
The value of CV of the retention time, the ratio of area chromatogram, and a peak high ratio of chromatogram at <10% for sample analysis of biological fluids. Further, the value of the asymmetry factor met the required value of <2.

### CONCLUSION

This study developed a method for the optimization of extraction conditions of OFX in blood plasma using Oasis hydrophilic and lipophilic balance SPE 1 cc, and further analysis using a HPLC-UV detector resulted in valid results. The results of validation methods met the following parameters: selectivity, repeatability, and linearity, limits of detection, LOQ, accuracy, precision, and suitability of the system. Since the method used had validity in accordance with the specified parameters, it can be used as a method for analyzing OFX in human blood plasma.

### REFERENCES