A simple HPLC–UV method for the determination of ciprofloxacin in human plasma

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A B S T R A C T
A rapid and sensitive HPLC–UV method for the determination of ciprofloxacin in human plasma is described. Protein precipitation with acetonitrile was used to separate the drug from plasma protein. An ACE® 5 C18 column (250 mm × 4.6 mm, 5 μm) with an isocratic mobile phase consisting of phosphate buffer (pH 2.7) and acetonitrile (77:23, v/v) was used for separation. The UV detector was set at 277 nm. The method was validated in the linear range of 0.05–8 μg/ml with acceptable inter- and intra-assay precision, accuracy and stability. The method is simple and rapid and can be used to quantify this widely used antibiotic in the plasma of patients suffering from Peripheral Arterial Disease.

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1. Introduction

Ciprofloxacin ([1-cyclopropyl-6-fluoro-4-oxo-7-((piperazin-1-yl)-quinoline-3-carboxylic acid]) is a 4-quinolone derivative, derived from nalidixic acid [1]. It provides effective treatment for a variety of infections particularly those of the urinary tract, respiratory tract, gastrointestinal tract, skin and soft tissues [2].

Its spectrum of activity and favourable pharmacological properties make ciprofloxacin useful in the treatment of diabetic foot infections [3]. Peripheral Arterial Disease (PAD) is a common cardiovascular complication in patients with diabetes and its presence increases the chance of treatment failure when dealing with infections such as foot infections [4]. The presence of significant PAD in an infected limb impairs delivery of the required dose of ciprofloxacin to the infected tissues [5]. A standard antibiotic dosage regimen may lead to sub-inhibitory concentrations at the target site. This decreases the effectiveness of antimicrobial therapy. In light of this, this study aims to develop and validate an innovative HPLC method for the quantification of ciprofloxacin in human plasma. This method will be subsequently used to quantify the concentration of ciprofloxacin in the peripheries of patients with PAD to establish if the dosage regimen given is sufficient to eradicate the infection at the target site.

HPLC can be used efficiently in the analysis of ciprofloxacin as it offers rapid results and is specific and sensitive [6]. Different types of detectors such as UV or fluorescence detectors can be coupled to HPLC. UV detectors are often preferred because they are cheaper and more easily available [7]. Mass spectrometry (MS) detectors can also be used. Although HPLC–MS offers excellent selectivity and sensitivity, it is relatively expensive instrumentation and skilled technical expertise is required [8]. As ciprofloxacin is a relatively polar compound, it is best separated from biological fluids using a technique such as protein precipitation rather than liquid–liquid extraction.

Chromatographic retention times of over 10 min for ciprofloxacin have been reported in previously published studies [9,10]. This makes the study less applicable for the analysis of a large number of samples. In addition, some of the reported methods involve the use of large volumes (≥1000 μl) of plasma/serum samples [9,11] rendering them unsuitable for repeat sampling where blood sample volumes can be limited.

2. Experimental

2.1. Reagents and standards

All liquids used were HPLC grade. Acetonitrile, orthophosphoric acid, ultrapure analytical grade type 1 water (r > 18 mΩ/cm) and hydrochloric acid were obtained from Fisher Scientific (Loughborough, Leicestershire, UK). Disodium hydrogen phosphate was

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obtained from Scharlau (Sentmenat, Spain). Standard ciprofloxacin, ofloxacin and sulfadimidine sodium powders were purchased from Sigma–Aldrich (Steinheim, Germany). Pooled drug-free human serum was obtained from Mater Dei Hospital, Malta.

2.2. Instrumentation

The study was carried out on a Varian® Pro Star HPLC unit consisting of an online degasser, column oven and UV–vis detector. Signals were registered using a Star® 800 Module Interface Box and processed using Galaxie® Workstation software. Signal quantification was carried out in the peak area mode. A XS104 Mettler Toledo analytical balance was used to weigh the analytes for the preparation of stock solutions and calibration standards. All solvent evaporation were carried out in a TurboVap® LV Automated Evaporation System.

2.2.1. Analytical and chromatographic conditions

Chromatographic separation was achieved on a reversed phase ACE® 5 C18 column (250 mm × 4.6 mm, 5 μm; Advances Chromatography Technologies, Aberdeen, Scotland) protected by a Agilent Pursuit 5 C18 Meta Guard® column (10 mm × 4.6 mm, 5 μm; Agilent Technologies, Amstelveen, Netherlands). Column and injection temperatures were both maintained at 25 °C. The system was operated isocratically at a flow rate of 1.5 ml/min. The sample was injected through a fixed sample loop having a volume of 50 μl. The UV detector was set at 277 nm.

2.3. Selection of the internal standard

Ofloxacin was initially chosen as the internal standard. However, when ciprofloxacin and ofloxacin were analysed together using the conditions described above, they both had a similar retention time of around 3 min. The percentage of acetonitrile was decreased from 30% to 26%, 25%, 24% and 23% in an attempt to better resolve the peaks given by the two compounds. This did not produce a considerable shift in the retention time of ofloxacin. The internal standard was subsequently changed to sulfadimidine sodium which has 2 pKa values of 2.65 ± 0.2 (pKα1) and 7.40 ± 0.2 (pKα2)[12,13], good UV absorption at 277 nm and similar chemical behaviour under the extraction and chromatographic conditions used in this study.

2.4. Preparation of the mobile phase

A 0.02 M phosphate buffer at pH 2.7 was prepared using disodium hydrogen phosphate and orthophosphoric acid. This was then eluted together with acetonitrile to make up a mobile phase of buffer and acetonitrile 77:23 (v/v). Liquids used for the mobile phase were kept in amber glass bottles. Fresh buffer was prepared daily.

2.5. Preparation of stock solutions

A 1 mg/ml ciprofloxacin stock solution was initially prepared in methanol. During analysis of this solution following storage for 1 week, 2 peaks were noted. When compared to previous analysis of the same solution, after 1 week the first peak which represented ciprofloxacin, decreased in size and a new peak was observed at a later retention time.

In response to this, the stock solution was prepared using 0.2 M hydrochloric acid. When the solution was analysed, peak shouldering was observed in the chromatogram. For this reason, ciprofloxacin was dissolved in the mobile phase. Working solutions were prepared from the stock solution by dilution with mobile phase. Sulfadimidine sodium was dissolved in HPLC-grade water to make up a stock solution of 1 mg/ml. All solutions were protected from light and were stored at 4 °C.

2.6. Sample preparation

Four hundred microlitres of plasma spiked with ciprofloxacin were transferred to 1.5 ml Eppendorf tubes. Thirty microlitres of internal standard (IS) working solution in water (100 μg/ml) were added to each tube. One drop of 10 M phosphate buffer (pH 2.7) was added and the tubes were vortex mixed for 3 min. Five hundred microlitres of ice cold acetonitrile were added using a glass syringe. The tubes were vortex mixed for a further 5 min. The samples were centrifuged at 3500 × g for 5 min. The supernatant was poured into 8 ml silanised glass tubes. The silanised tubes were placed in a TurboVap Concentrator® with the water bath set at 50 °C for 20 min. The dried residue was reconstituted with 100 μl mobile phase, vortex mixed for 3 min and re-centrifuged at 15,000 rpm for 3 min. Fifty microlitres of the clear supernatant were injected into the HPLC unit.

2.7. Assay validation

In order to confirm the suitability of the method for its intended use, it was validated for specificity, linearity, precision, accuracy, limit of quantification, limit of detection and stability according to the International Conference on Harmonisation (ICH) guidelines [14].

2.7.1. Specificity

The specificity of the method was determined by analysing 5 human blank plasma samples.

2.7.2. Linearity

Calibration standards were prepared from the ciprofloxacin stock solution at 7 concentration levels ranging from 0.05 to 8 μg/ml. This incorporates the clinically relevant plasma concentration range [15,16]. Peak area ratios (ciprofloxacin/IS) were plotted against the corresponding ciprofloxacin concentrations in plasma. Least-squares linear regression analysis of the calibration data was performed using the linear equation $y = mx + c$.

2.7.3. Precision

Intraday precision was evaluated by analysing plasma aliquots of the calibration standards in 5 replicates on the same day. The inter-assay precision was determined by analysing each calibration sample once for 4 consecutive days. Intra-assay and inter-assay precision were expressed as the percentage relative standard deviation (RSD).

2.7.4. Accuracy

Accuracy was expressed as the percentage recovery and was calculated as the measured value/theoretical value × 100. Analyte recovery was tested in triplicate for 3 concentrations (0.5, 2 and 6 μg/ml).

3. Results and discussion

3.1. Stock solution preparation

Ciprofloxacin is only slightly soluble or insoluble in water [17]. It was initially dissolved in methanol. The second peak eluting after the peak of ciprofloxacin was attributed to the methyl ester of ciprofloxacin, following an esterification reaction with the methanol in solution during storage. This has been previously documented [8,18]. To resolve this problem, the stock solution was
prepared in 0.2 M hydrochloric acid. When this solution was analysed using the same conditions, peak shouldering was noted. This was attributed to the low pH present which affected pH control during chromatography. The stock solution was consequently prepared in the mobile phase.

3.2. Selection of the internal standard

Other fluoroquinolones are often used as internal standards for ciprofloxacin, due to similarities in chromatographic behaviour [8,19,20]. Initially, ofloxacin was chosen as the internal standard but both analytes eluted at similar retention times, making resolution poor and quantification difficult. Decreasing the amount of acetonitrile in the mobile phase should favour the interaction of ofloxacin to the hydrophobic stationary phase relative to the mobile phase and increase the retention time [21]. In this case this did not produce a considerable shift in the retention time of ofloxacin and the internal standard was subsequently changed to sulfadimidine sodium. The use of this internal standard for ciprofloxacin has not yet been documented in literature. This standard eluted well after ciprofloxacin and did not affect resolution.

3.3. Sample preparation

Sample preparation is used to remove plasma proteins and other compounds from plasma that can potentially interfere with the analyte of interest and damage the analytical column [8].

3.3.1. Protein precipitation

Protein precipitation with acetonitrile or methanol is the most commonly used sample pre-treatment method for compounds such as ciprofloxacin [22]. As the polarity of an organic solvent increases, it becomes a less effective precipitating agent [23]. Methanol is more polar than acetonitrile and is therefore expected to be less effective at precipitating proteins. Protein precipitation with acetonitrile was the most commonly used sample preparation method in studies related to ciprofloxacin [8,24-26].

3.3.2. Optimisation of acetonitrile: plasma ratio

In the present study, protein precipitation was best achieved using 500 μl of acetonitrile and 400 μl of plasma. A combination of 1 ml acetonitrile and 400 μl of plasma was also used but this resulted in the formation of a cloudy supernatant following centrifugation. The use of equal volumes of acetonitrile and plasma can lead to precipitation of about 95% of the proteins [27]. Haeseker, Khan and Weber made use of equal volumes of acetonitrile and plasma when precipitating plasma proteins for the analysis of ciprofloxacin [28,11,26]. According to Neckel, the addition of 1.5 volumes of acetonitrile to one volume of plasma is sufficient to remove 99.4% of the proteins [29]. According to Venn, the use of large volumes of organic solvents adds to the volume to be evaporated, increasing the assay time [7]. Chromatography can be altered if too large a volume of a strong solvent is injected onto an HPLC column, causing tailing or extensive peak broadening. Retention times can also be changed if solvents are not evaporated off completely from the sample [30].

3.3.3. Second centrifugation step

Direct injection of the supernatant obtained after protein precipitation by acetonitrile was described in previous studies [11,26,28]. This was tried in the present study and 50 μl of supernatant obtained after protein precipitation were injected into the HPLC unit. However, this blocked the guard column. An approach to inject a clearer supernatant into the HPLC unit was sought. Espinosa described filtering the clear supernatant through a 0.45 μm syringe adapter before injection [31]. However, in the present study loss of the analyte of interest was observed when this was done.

A second centrifugation step after reconstituting the dried residue with the mobile phase and vortex mixing was consequently added prior to injection onto the HPLC unit. This resulted in the analysis of a clearer supernatant.

3.4. Adsorption and carry over

Carboxylic acid functional groups tend to react with the surface of laboratory glassware, which is slightly alkaline. This results in adsorption and a consequent loss of analyte affecting recovery and reproducibility. To prevent sample loss through adsorption, glassware can be silanised. The coating provides a barrier between the contents and the glass, eliminating active sites on the glass that could potentially react with its contents [32]. In the present study the glass tubes used for protein precipitation were silanised using an in-house silanisation procedure. Glass tubes were left to soak for 30 min in dimethylchlorosilane and washed 3 times with methanol. The tubes were then dried in an oven at 110 °C. The use of silanised glass tubes decreased the interaction of the polar ciprofloxacin with glass and increased reproducibility and recovery (Table 1).

The syringe used to inject the samples into the HPLC was rinsed well with acetonitrile following use to avoid carryover [33]. The use of polypropylene containers, whenever possible, was preferred. To prevent carryover of ciprofloxacin onto the analytical column, a blank acetonitrile injection was made following every injection of ciprofloxacin.

3.5. Assay validation

3.5.1. Specificity

Specificity was confirmed by the absence of peaks at the retention times of ciprofloxacin and sulfadimidine (Fig. 1).

3.5.2. Linearity

The calibration curve for ciprofloxacin was linear in the concentration range of 0.05–8 μg/ml in human plasma, with an average r value of 0.999 (n = 6) (Fig. 2). The mean equation of the regression line derived from 6 replicates was y = 32.6508x + 0.0337.

Table 1

<table>
<thead>
<tr>
<th>Silanised tubes</th>
<th>Non-silanised tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated quantity (μg/ml)</td>
<td>Percentage recovery</td>
</tr>
<tr>
<td>3.90</td>
<td>97.50</td>
</tr>
<tr>
<td>3.87</td>
<td>96.75</td>
</tr>
<tr>
<td>3.82</td>
<td>95.50</td>
</tr>
<tr>
<td>3.73</td>
<td>93.25</td>
</tr>
<tr>
<td>4.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Average percentage recovery (N = 5) 96.90 86.10
Relative standard deviation 1.16 3.59
Fig. 1. Chromatogram of ciprofloxacin (7 μg/ml); retention time = 3.26 min and sulfadimidine sodium; retention time = 4.92 min in plasma.

Table 2
Intraday precision values for the determination of ciprofloxacin in human plasma (N = 5).

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Mean of 5 replicates (μg/ml)</th>
<th>Standard deviation (μg/ml)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>2.6287</td>
<td>0.0296</td>
<td>1.1268</td>
</tr>
<tr>
<td>6</td>
<td>1.9878</td>
<td>0.0463</td>
<td>2.3273</td>
</tr>
<tr>
<td>4</td>
<td>1.3555</td>
<td>0.0158</td>
<td>1.688</td>
</tr>
<tr>
<td>2</td>
<td>0.7283</td>
<td>0.0281</td>
<td>3.8570</td>
</tr>
<tr>
<td>0.5</td>
<td>0.2234</td>
<td>0.0197</td>
<td>8.8059</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0393</td>
<td>0.0018</td>
<td>0.0455</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0200</td>
<td>0.0026</td>
<td>13.0820</td>
</tr>
</tbody>
</table>

Table 3
Interday precision values for the determination of ciprofloxacin in human plasma (N = 5).

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Mean of 5 replicates (μg/ml)</th>
<th>Standard deviation (μg/ml)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>2.4208</td>
<td>0.0242</td>
<td>0.9989</td>
</tr>
<tr>
<td>6</td>
<td>1.9556</td>
<td>0.0937</td>
<td>4.7934</td>
</tr>
<tr>
<td>4</td>
<td>1.2838</td>
<td>0.0268</td>
<td>2.0837</td>
</tr>
<tr>
<td>2</td>
<td>0.6916</td>
<td>0.0285</td>
<td>4.1180</td>
</tr>
<tr>
<td>0.5</td>
<td>0.1719</td>
<td>0.0077</td>
<td>4.4613</td>
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<tr>
<td>0.1</td>
<td>0.0385</td>
<td>0.0023</td>
<td>6.0831</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0187</td>
<td>0.0023</td>
<td>12.0374</td>
</tr>
</tbody>
</table>

Table 4
Extraction recovery for ciprofloxacin from spiked plasma (N = 4).

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Mean calculated quantity of 3 replicates (μg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>5.7667</td>
<td>96.1117</td>
</tr>
<tr>
<td>2</td>
<td>1.9800</td>
<td>99.0000</td>
</tr>
<tr>
<td>0.5</td>
<td>0.4500</td>
<td>90.0000</td>
</tr>
</tbody>
</table>
3.5.3. **Precision**

The percentage relative standard deviation (RSD) of the quantities of ciprofloxacin detected during the intraday study was found to range between 0.05 and 8.94 (Table 2).

Interday precision RSD values were all below 12.59% (Table 3).

3.5.4. **Accuracy**

The quantitative recoveries of ciprofloxacin in plasma achieved ranged from 90.0006% to 96.1117%. The mean recoveries for all three concentrations analysed are given in Table 4.

3.5.5. **Limit of detection and limit of quantification**

The LOD was 0.01 µg/ml and the LOQ was 0.05 µg/ml.

3.6. **Application of the method**

To demonstrate the reliability of this method for the study of ciprofloxacin in human plasma, this assay was applied clinically to measure the concentrations of ciprofloxacin in the plasma of ten patients suffering from peripheral arterial disease. This was done following approval from the University Research Ethics Committee (UREC).

Patients were being given ciprofloxacin intravenously (400 mg bd) 30 min prior to having a debridement or amputation procedure. Serum samples were collected from these patients at the start of the procedure. A representative chromatogram from an extracted plasma sample from a patient is shown in Fig. 3. There were no interfering endogenous peaks (Table 5).

3.7. **Results**

Table 5 shows the stability of ciprofloxacin after 7 and 30 days. The concentration values were within the limit of quantification (LOQ), indicating that the method is suitable for clinical use.

3.8. **Discussion**

This study demonstrated that the developed method is suitable for clinical use in patients suffering from peripheral arterial disease. The low RSD values and the quantitative recoveries indicate high precision and accuracy of the method. The method was able to detect and quantify ciprofloxacin concentrations in serum, which is important for diagnosing and monitoring the effectiveness of the drug in the treatment of infections.

4. **Conclusion**

This innovative method offers several advantages for assaying ciprofloxacin in human plasma including simplicity, cost effectiveness, good precision and accuracy with high sensitivity and selectivity. The use of sulfadimidine sodium has not been previously reported and provided good resolution from the analyte of interest. Moreover, the method requires only a small volume (400 µl) of plasma and has a short run time (5 min). This method will be further modified and used to quantify ciprofloxacin concentration in tissue and to compare the concentration of ciprofloxacin present in serum with the concentration in the ischaemic peripheries of patients suffering from PAD. This data would allow selection of dosage regimens which achieve adequate concentrations at the target site, ensuring eradication of the causative pathogens and a possible decrease in the need for amputations due to poorly controlled infections.

**Table 5**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>After 7 days (% RSD)</th>
<th>After 30 days (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>7.3077</td>
<td>0.4963</td>
</tr>
<tr>
<td>2</td>
<td>1.733</td>
<td>3.7097</td>
</tr>
<tr>
<td>8</td>
<td>6.7406</td>
<td>2.8664</td>
</tr>
</tbody>
</table>

**References**


