Validation and Application of a New Optimized RP-HPLC-Fluorescent Detection Method for Norfloxacin

Mahmood Ahmad, Ghulam Murtaza, Sonia Khiljee and Muhammad Asadullah Madni

Abstract—A new reverse phase-high performance liquid chromatography (RP-HPLC) method with fluorescent detector (FLD) was developed and optimized for Norfloxacin determination in human plasma. Mobile phase specifications, extraction method and excitation and emission wavelengths were varied for optimization. HPLC system contained a reverse phase C18 (5 μm, 4.6 mm × 150 mm) column with FLD operated at excitation 330 nm and emission 440 nm. The optimized mobile phase consisted of 14% acetonitrile in buffer solution. The aqueous phase was prepared by mixing 2g of citric acid, 2g sodium acetate and 1 ml of triethylamine in 1 L of Milli-Q water was run at a flow rate of 1.2 mL/min. The standard curve was linear for the range tested (0.156–20 μg/mL) and the coefficient of determination was 0.9978. Aceclofenac sodium was used as internal standard. A detection limit of 0.078 μg/mL was achieved. Run time was set at 10 minutes because retention time of norfloxacin was 0.99 min. which shows the rapidness of this method of analysis. The present assay showed good accuracy, precision and sensitivity for Norfloxacin determination in human plasma with a new internal standard and can be applied pharmacokinetic evaluation of Norfloxacin tablets after oral administration in human.

Keywords—Norfloxacin, Aceclofenac sodium, Method optimization, RP-HPLC method, Fluorescent detection, Calibration curve.

I. INTRODUCTION

Norfloxacin is a new broad-spectrum antibacterial agent that is structurally related to nalidixic acid [1]. Its chemical structure is given in Figure-1. Norfloxacin exhibits greater antibacterial activity against gram -positive and gram-negative bacteria than other nalidixic acid analogs and exhibits greater activity against pseudomonas aeruginosa than gentimycin [2]. This is because the fluorine atom at the 6 position provides increased potency against gram-negative organisms and the piperazine moiety at the 7 position is responsible for anti-pseudomonal activity [3, 4, 5]. This potent antibiotic is still one of the most prescribed chemotherapeutic agent against many infections especially urinary tract infection [5].

Fig. 1 Structural formula of Norfloxacin

In Pakistan, yet no study has been conducted on pharmacokinetics and bioavailability of Norfloxacin. Consequently, it was very important to conduct the pharmacokinetics and bioavailability studies of Norfloxacin in the local population due to the effect of race. As a part of above mentioned study, a new valid HPLC method was therefore, developed in our laboratory using Aceclofenac sodium as internal standard. Moreover, the literature survey denies the use of Aceclofenac sodium as internal standard in any earlier study.

II. MATERIALS AND METHODS

A. Chemicals and Reagents

Norfloxacin was gifted by Zhejiang Medicine & Health Products, China. Aceclofenac sodium was gifted by Xenon Pharmaceuticals, Pakistan. All other reagents (analytical grade or chromatography grade) were purchased from commercial sources and were used as received without further purification.

B. Instrumentation

High-Performance Liquid Chromatographic System (Agilent Technologies, 1200 Series, USA) consisted of a water solvent delivery system, an iso pump (G1310A) and a rheodyne attached with 20 μl loop. The above system was controlled by the ChemStation software equipped with a fluorescent detector (FLD, G1321A).
C. Chromatographic Conditions
A Reverse phase C18 (5μm, 4.6mmx150mm) (Agilent, USA) was used. HPLC system was operated at room temperature. The mobile phase consisted of 14% acetonitrile in buffer solution. The aqueous phase was prepared by mixing 2g of citric acid, 2 g sodium acetate and 1ml of triethylamine in 1 liter of Milli-Q water (42). was prepared, filtered through Cellulose Acetate Filter (0.45 μm pore size, Sartorius, Germany) and degassed by sonicator (Elma, Germany) at 70 Hz before use. Mobile phase was delivered at a rate of 1.2 ml/min. Injection volume was 20 μl. FLD was operated at excitation wavelength 330 nm and emission wavelength 440 nm.

D. Preparation of Samples
Stock solutions of norfloxacin and Aceclofenac sodium were prepared fresh daily by dissolving 1 mg of drug in 1 ml water. Blank plasma was spiked with Norfloxacin drug solutions to give calibration curve concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156 μg/ml. Aceclofenac sodium (5 μg/ml) was also added in each dilution. Extraction procedure was same as described below. Injections of 20 μl were injected and spectra were taken of each concentration. The peak areas were noted for each concentration and calibration curve was drawn. The intra-day (within-run) and inter-day (between-run) accuracy and precision of method were determined on three separate days.

E. Optimization Studies
Following parameters were varied to produce optimum separation of norfloxacin from plasma: (1) choice of solvent for the preparation of stock solution (2) Nature and composition of mobile phase (3) pH of the mobile phase (3.0; 4.0; 5.0), (4) excitation and emission wavelengths of FLD, (5) sample concentrator temperature (6) flow rate of mobile phase (7) choice of protein extracting agent and its amount.

F. Optimum Sample Clean up Procedure
A 200 μl plasma sample was taken in a glass-stoppered tube and 500 μl of acetonitrile was added to precipitate the plasma proteins; the mixture was shaken for 1 min and centrifuged at 4000 rpm for 15 min .The supernatant plasma layer was stored at -20 °C until assayed. The protocol of study was approved by the committee for research of the Islamia University of Bahawalpur, Bahawalpur, Pakistan.

III. RESULTS AND DISCUSSION

A. Optimization Studies
Norfloxacin peak shape problems are common which can be corrected by certain steps e.g. variation in mobile composition, excitation and emission wavelengths etc.

B. Mobile Phase
In the search of an appropriate mobile phase, following mobile phases are tried; 1. Phosphoric acid solution (1:100, v/v) and acetonitrile (850:150). 2. Phosphoric acid solution (1:100, v/v) and acetonitrile (150:850). 3. Isopropyl alcohol – citrate buffer 0.4 M - Water, (7:15:78, v/v). 4. Methanol : 0.1 M perchloric acid (30:70, v/v) and 0.02M triethylamine. 5. Methanol : 0.1 M perchloric acid (30:70, v/v) and 0.02M triethylamine. 6. Monobasic potassium phosphate 0.05 M : ion-pairing reagent (pH = 3.0, adjusted with 85% phosphoric acid) (80:20, v/v). 7. Acetonitrile : tetra-butyl ammonium hydroxide : phosphate buffer 0.05 M (pH = 3.0, adjusted with 85% phosphoric acid) (5:15:80). 8. Acetonitrile : phosphate buffer 0.05 M (pH = 7.0 adjusted with 85% phosphoric acid) (20:80, v/v). 9. Acetonitrile : acetate buffer solution 0.07 (14:86, v/v). The aqueous phase was prepared by mixing 2 g of citric acid, 2 g sodium acetate and 1 ml of triethylamine in 1 L of Milli-Q water.

It was observed that non-buffer mobile phases produced low sensitivity as compared buffer solvent systems. Mobile phase 2 and 5 produced peaks with low separation factor while mixture 1 and 7 exhibited high retention time with comparatively low sensitivity. Mobile phase 9 produced well resolved definite peaks (Rs > 1.5) with low retention time. Therefore this mixture was set as optimum solvent system for further study.

Similarly, various excitation and emission wave lengths were also tried but separation at excitation and emission wavelengths of 330 and 440 nm was optimum so it was
adopted for further study. Methanol, acetonitrile, di-(2-ethylhexyl) phosphate and perchloric acid with different ratios were tried to get best extraction of proteins. However, the clean up procedure mentioned in materials and methods was found optimum.

C. Validation of Method

The developed method with all optimum conditions mentioned in above section was validated. Blank plasma was spiked with Norfloxacin drug solutions to give calibration curve concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156 \( \mu \)g/ml and calibration curve was drawn using Microsoft excel (Figure-2).

\[
y = 49.492x + 21.751 \\
R^2 = 0.9978
\]

Good linearity was observed with \( R^2 = 0.9978 \) in a range of 20-0.156 \( \mu \)g/ml. This method was repeated in different atmospheric conditions by different researchers but found no significant variation in the results. Therefore, it shows good robustness and ruggedness. The lowest limit of quantification and detection were 0.156 \( \mu \)g/ml and 0.078 \( \mu \)g/ml, respectively. The methods presented by Hermann and Christian, (1998), Pauliukonis et al., (1982) and Samanidou et al., (2003) showed lowest limit of quantitation as 31, 50 and 30 ng/ml, respectively [6-8]. These results show good sensitivity of this HPLC method for the quantification of norfloxacin in human plasma. The inter- and intra-run precision, calculated from quality control samples, was less than 6.2%. The accuracy determined from quality samples was within \( \pm 2.5\% \). A typical chromatogram showing norfloxacin and internal standard is given in Figure-3. The percentage recovery of this method was 91.17%. The method found in literature also show similar norfloxacin recovery (%) as 88.3%, 85% and 97% by Hermann and Christian, (1998), Pauliukonis et al., (1982) and Samanidou et al., (2003), respectively. The extraction efficiency of this method for Aceclofenac sodium was about 83%. The retention time of norfloxacin and internal standard in this method was 0.99 min and 0.43 min, respectively. It shows rapidness of norfloxacin analysis by this method as compared to previous ones due to short analysis time [9].

D. Application to Pharmacokinetic Study

This rapid optimized and validated method was applied to pharmacokinetic study of norfloxacin in human. For this purpose, norfloxacin 400 mg tablets (Noroxin 400 mg, MSD) were ingested to 18 human volunteers and their blood samples were collected at regular intervals, centrifuged and analyzed on HPLC after extraction of plasma samples. The areas of various peaks at different time intervals were plotted against time to plasma drug concentration profiles (Figure-4). From this data, the pharmacokinetic parameters were also determined using “Kinetica”, shown in Table-1.

IV. CONCLUSION

A new specific RP-HPLC method has been developed, optimized and validated for norfloxacin estimation in human plasma using FLD. Results showed that the mobile phase composition and excitation and emission wave lengths of FLD affect method reproducibility and quantitative determination of the norfloxacin. The method was found to be linear over an analytical range of 0.156-20 \( \mu \)g/mL with LOD = 0.078 ng/mL and LOQ = 0.156 \( \mu \)g/mL respectively. Aceclofenac sodium eluted earlier than norfloxacin which is a property of a good internal standard. This method also showed good accuracy and precision. Thus it can be concluded that this method can be applied in routine drug monitoring and pharmacokinetic studies of norfloxacin.
### TABLE I COMPARISON OF MEAN ± SEM OF BIOAVAILABILITY AND PHARMACOKINETIC PARAMETERS OF NOROXIN®8400 MG-MSD IN NORMAL SUBJECTS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Noroxin-MSD (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (µg/ml)</td>
<td>1.421 ± 0.055</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.500 ± 0.087</td>
</tr>
<tr>
<td>$AUC$ (µg h/ml)</td>
<td>5.409 ± 0.156</td>
</tr>
<tr>
<td>$AUMC$ (µg h²/ml)</td>
<td>26.166 ± 0.766</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>4.840 ± 0.044</td>
</tr>
<tr>
<td>$K_e$ (h⁻¹)</td>
<td>0.207 ± 0.002</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>3.354 ± 0.030</td>
</tr>
<tr>
<td>$V_d$ (L/Kg)</td>
<td>361.316 ± 11.458</td>
</tr>
<tr>
<td>$V_{ss}$ (L/Kg)</td>
<td>15.423 ± 0.426</td>
</tr>
<tr>
<td>$Cl$ (ml/h/Kg)</td>
<td>74.616 ± 2.103</td>
</tr>
</tbody>
</table>

* = Significant difference (P≤0.05)
ns = Non significant difference (P>0.05)

### REFERENCES


