SIMPLE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR DETERMINATION OF NORFLOXACIN IN PLASMA AND APPLICATION IN BIOEQUIVALENCE STUDY

GABRIEL ONN KIT LOH, KAI BIN LIEW, KOK KHIANG PEH* AND YVONNE TZE FUNG TAN,
School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia. Email: kkepb@usm.my

Received: 13 Feb 2012, Revised and Accepted: 15 Mar 2012

ABSTRACT
A simple, sensitive and selective HPLC method with fluorescence detection for determination of norfloxacin in human plasma was developed. Protein precipitation of plasma samples was performed using perchloric acid 70%. Chromatographic separation of norfloxacin was achieved using Agilent Eclipse Plus C18 column (250 x 4.6 mm). The mobile phase was comprised of 0.01 M potassium dihydrogen phosphate and acetonitrile (75:25, v/v) adjusted to pH 2.7 with phosphoric acid. The analysis was run at a flow rate of 0.8 mL/min with an injection volume of 10 µL. The detector was operated at an excitation wavelength of 310 nm and an emission wavelength of 450 nm. The calibration curve was linear over a concentration range of 30 – 2500 ng/mL. Intra-day and inter-day precision and accuracy values were below 15%. The limit of quantification was 30 ng/mL and the mean recovery was 94%. Freeze-thaw, short-term, long-term and post-preparative stability studies showed that norfloxacin in plasma sample was stable. The method was successfully applied to analyse the norfloxacin concentration in plasma samples from bioequivalence studies.

Keywords: Norfloxacin, HPLC, Human plasma

INTRODUCTION
Norfloxacin is a synthetic fluorinated analog of nalidixic acid. It is active against gram-positive and gram-negative bacteria.1 Norfloxacin blocks bacterial DNA synthesis by inhibiting bacterial topoisomerase II, which is also known as DNA gyrase and topoisomerase IV. Inhibition of DNA gyrase prevents normal transcription and replication while inhibition of topoisomerase IV interferes with separation of replicated chromosomal DNA into the respective daughter cells during cell division.2 After oral administration of norfloxacin, it is well absorbed from gastrointestinal tract into the systemic circulation with oral bioavailability of around 80% and reaches the maximum concentration in 1-2 hour. Approximately 30% of norfloxacin is excreted as un-metabolized drug in the urine.1,2 Various HPLC methods have been reported for the determination of norfloxacin in biological fluids. Liquid-liquid3 and solid phase extraction methods were used in sample treatment. However, the extraction methods are generally tedious and time consuming. Hussain et al4 carried out an additional extraction step with chloroform following protein precipitation with trichloroacetic acid. Mascher and Kikut3 used acetonitrile, which was comparatively less effective than trichloroacetic acid for protein precipitation, as the plasma samples were found dirty.

This paper describes a rapid, simple, selective and sensitive HPLC method for the determination of norfloxacin in human plasma. The method involved protein precipitation of the plasma samples using perchloric acid followed by quantification using HPLC with fluorescent detection. The method was also applied to analyse norfloxacin concentration in plasma samples from bioequivalence studies. Bioequivalence of a drug is achieved if the test product’s rate and extent of absorption is not statistically significant different from those reference product when administered at the same molar dose.6 Pharmacokinetic studies are required to determine the ability of a drug to achieve and maintain concentrations that maximize their efficacy and safety.9

MATERIALS AND METHODS
Reagents and Chemicals
Norfloxacin working standard was supplied by Malaysian Pharmaceutical Industries (Penang, Malaysia). Perchloric acid 70%, potassium dihydrogen phosphate and phosphoric acid are of analytical grades were purchased from R & M (Essex, United Kingdom). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used were of analytical grade.

Equipment
The HPLC system was comprised of a Shimadzu pump (LC-10AT vp/FCV-10AL), an auto-injector (SL-10AD) and a fluorescence detector (VP series, Kyoto, Japan). Data processing was performed using LC solution software (Class VP). Chromatography separation of norfloxacin was achieved using Eclipse Plus C18 (250 x 4.6 mm ID, 5 µm) column (Agilent, USA) fitted with analytical guard column (Zorbax Eclipse Plus, Agilent, USA) packed with replaceable C-18 cartridge (12.5 x 4.6 mm ID, 5 µm).

Chromatography
The mobile phase consisted of 0.01 M potassium dihydrogen phosphate and acetonitrile (75:25, v/v) adjusted to pH 2.7 with phosphoric acid. The analysis was run at ambient room temperature (26°C) with a flow rate of 0.8 mL/min and an injection volume of 10 µL. The detector was operated at an excitation wavelength of 310 nm and an emission wavelength of 450 nm.

Preparation of Standard Drug Solutions
Stock drug solution of 20 ng/mL was prepared by dissolving 10 mg of norfloxacin in 500 mL of mobile phase. The working standard drug solutions of 300 ng/mL, 2500 ng/mL and 15000 ng/mL were prepared by serial dilution of the stock drug solution. All stock solutions were stored at 2-8 ºC.

Preparation of Quality Control Samples
Three quality control (QC) plasma samples at concentrations of 120 ng/mL, 750 ng/mL and 2000 ng/mL were prepared by spiking into 250 µL blank plasma with appropriate working standard drug solution.

Preparation of Standard Plasma Samples
Standard plasma samples ranged from 30 to 2500 ng/mL were prepared by spiking 250 µL blank human plasma samples with suitable working standard drug solution.

Plasma Sample Treatment
An aliquot of 250 µL of norfloxacin spiked plasma samples were measured accurately into 2.0 mL Eppendorf®tubes, followed by an addition of 10 µL of perchloric acid (70%) to precipitate the plasma protein. The mixture was vortexed for one minute and centrifuged at 12000 rpm for 10 minutes. The supernatant was collected and
transferred into autosampler vials. Plasma sample of 10 µL was injected into the HPLC column.

**Method Validation**

The method was validated with respect to selectivity, linearity, precision, accuracy, recovery, limit of quantification (LOQ), and stability. The quality control samples at concentrations of 120, 750 and 2000 ng/mL as well as 30 ng/mL (LOQ) were used to determine the within-day and between-day precision, accuracy and recovery of the assay method. The freeze and thaw stability, short-term temperature stability, post-preparative stability and long term stability were carried out at concentrations of 120 and 2000 ng/mL while the stock solution stability was performed at concentration of 2000 ng/mL.

**Selectivity**

The selectivity of the method was determined to ensure the analyte peak was well separated and free from interference of endogenous compounds in the plasma, using blank (drug free) plasma samples obtained from 6 healthy volunteers.

**Linearity**

Standard plasma samples with concentration range of 30-2500 ng/mL were prepared by spiking different concentration of norfloxacin working standard solutions into blank human plasma. Standard calibration curve was constructed by plotting the peak height of norfloxacin against the corresponding concentrations of standard plasma samples. Linear regression equation was applied and correlation coefficient was determined. The linearity was determined from six standard calibration curves (n=6) over a concentration range of 30 – 2500 ng/mL (R², y = mx + c).

**Precision and Accuracy**

For inter-day precision and accuracy, six replicates of each analyte concentrations were injected over six consecutive days, while for intra-day precision and accuracy, six replicates of each analyte concentrations were injected on the same day. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for LOQ where it should not exceed 20% of the CV. For accuracy, the mean value should be within 15% of the actual value except at LOQ where it should not deviate more than 20%.

**Relative Recovery**

The samples were prepared as described in Section of preparation of plasma samples. The recovery was calculated by comparing the peak height obtained from the plasma sample after treatment, with that of the aqueous drug solution of corresponding concentration. Each concentration was analyzed six times.

**Limit of Quantification (LOQ)**

The limit of quantification was determined as the lowest concentration of norfloxacin in the standard calibration curve, which could be quantified with a value of bias below 20% and a signal to noise ratio of at least 5.

**Stability Studies**

**Freeze and Thaw Stability**

The analyte stability was determined after three freeze and thaw cycles. Three aliquots at each of the low and high concentrations were stored at -20°C for 24 hours and thawed unassisted at ambient room temperature of 26°C. When completely thawed, the samples were refrigerated for 24 hours under the same conditions. The freeze-thaw cycles were repeated two more times, and analyzed after the third cycle.

**Short Term Stability**

Three aliquots of each of the low and high concentrations were thawed unassisted at ambient at room temperature of 26°C and kept at this temperature for 8 hours (based on the expected duration that samples might be maintained at room temperature in the present study) and analyzed.

**Post-preparative Stability**

The stability of the processed samples, including the residence time in the auto-sampler was determined. The stability of norfloxacin was assessed over the anticipated run time of 8 hours for the batch size.

**Long Term Stability**

Three aliquots of each of the low and high concentrations of norfloxacin stored at -20°C over a period of three months were analyzed.

**Stock Drug Solution Stability**

The stability of stock solution of drug was evaluated at ambient room temperature of 26°C for 8 hours.

**Application in Bioequivalence Study**

The method was used to quantify norfloxacin concentration in human plasma samples of 18 healthy volunteers where the number of subjects required to conduct a BE study is 12; administered orally with 400 mg of norfloxacin in a bioequivalence study comparing a generic tablet (Norgan, Malaysian Pharmaceutical Industries, Malaysia) and an innovator tablet (Norflox, Merck Sharp & Dohme Limited, United Kingdom).

**RESULTS AND DISCUSSION**

**Method development and sample preparation**

In sample preparation, methanol, acetonitrile and perchloric acid were attempted for protein precipitation. Plasma samples treated with methanol and acetonitrile were found to be cloudy. In addition, a volume of at least 500 µL of methanol and acetonitrile was required for 250 µL of plasma samples to give a clear sample but this method reduced the sensitivity of the method due to dilution of the solvent. In the present study, the use of 10 µL of perchloric acid (70% w/v) was sufficient to precipitate the protein of 250 µL of plasma sample, to produce a clean plasma sample and the chromatogram.

Internal standard was not used in the present study since there was no extraction process involved.

The composition and pH of the mobile phase were critical. Norfloxacin peak was eluted faster with an increase in acetonitrile content. However, when the acetonitrile content was increased to 30%, norfloxacin peak was eluted earlier in 3.0 min and overlapped with the endogenous compound. At acetonitrile content of 20%, norfloxacin peak was eluted at retention time of 6 min. Hence, the use of 25% of acetonitrile was found to be optimum with the retention time of approximately 4.0 min. The retention time and peak shape of norfloxacin were also affected by the pH of mobile phase. At pH above 3, the analyte peak shape became broad and less symmetry. Moreover, the retention time was extended. At pH below 2.7, the peak shape was distorted. Hence, the mobile phase of 0.01 M potassium dihydrogen phosphate and acetonitrile (75:25, v/v) at pH 2.7 was found to be optimum.

**Selectivity**

The chromatograms of blank plasma and norfloxacin spiked plasma at 2000 ng/mL are shown in Fig 1 and 2. It can be seen that the peak of analyte was well resolved and free of interference from any endogenous compounds of the plasma.

**Linearity**

The standard calibration curve was linear over a concentration range of 30 – 2500 ng/mL. The regression line could be described by, y = 17.975 (± 0.761) x + 19.854 (± 107.199), with a correlation coefficient was greater than 0.999.

**Precision and Accuracy**

The inter-day and intra-day precision and accuracy results are given in Table 1. The inter-day precision results expressed as coefficient of variation (% CV) were less than 10.6 % and the accuracy values as percent error (%) were less than 6.9%. On the other hand, the intra-day precision values were less than 8.1% and the accuracy values were less than 7.7%. The results indicate that the method is precise and accurate.

Fig. 1: Chromatogram of blank plasma

Fig. 2: Chromatogram of plasma containing 2000ng/mL

Table 1: Intra-day and inter-day precision and accuracy data

<table>
<thead>
<tr>
<th>Known concentration spiked (ng/mL)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration found (ng/mL)</td>
<td>Precision (% RSD)</td>
</tr>
<tr>
<td>30</td>
<td>29.50 (± 1.64)</td>
<td>5.55</td>
</tr>
<tr>
<td>120</td>
<td>119.15 (± 2.42)</td>
<td>2.03</td>
</tr>
<tr>
<td>750</td>
<td>728.28 (± 58.29)</td>
<td>8.00</td>
</tr>
<tr>
<td>2000</td>
<td>1846.19 (± 37.12)</td>
<td>2.01</td>
</tr>
</tbody>
</table>

Mean ± standard deviation, n = 6.

Relative Recovery

The mean recovery value of 97.54 ± 5.41% was obtained at 30 ng/mL, 98.44 ± 2.00% at 120 ng/mL, 92.45 ± 7.40% at 750 ng/mL, and 86.94% ± 1.75 at 2000 ng/mL. Hence, the method shows an average recovery of above 90%.

Limit of Quantification (LOQ)

The LOQ was found to be 30 ng/mL. The level is sufficient to quantify norfloxacin concentration in plasma samples of bioequivalence and pharmacokinetic studies.

Stability Study

Table 2 shows the results of stability studies of norfloxacin in plasma. Two concentrations were used in stability studies, 120 and 2000 ng/mL, which were defined as low and high concentration respectively. After three freeze and thaw cycles, the norfloxacin spiked plasma samples had value of 98.17 ± 1.36 % for 120 ng/mL and 90.32 ± 2.43 for 2000 ng/mL. The results of short term stability showed mean value of 95.16 ± 5.62% at 120 ng/mL and 98.69 ± 2.41% at 2000 ng/mL. The long term stability results were 98.79 ± 5.84% at 120 ng/mL and 96.07 ± 1.93% at 2000 ng/mL. The post-preparative stability results were 105.83 ± 10.57% at 120 ng/mL and 101.81 ± 2.77% at 2000 ng/mL. The stock solution was stable at ambient room temperature for more than 6 hours with mean value of 100.97 ± 0.14% at 20000 ng/mL. The stability results showed that norfloxacin in plasma was stable under the study condition.

Application in Bioequivalence Study

Fig 3 shows the mean plasma concentration-time curve of norfloxacin of 18 volunteers after oral administration of 400 mg of norfloxacin of Norgan® and Norflox® tablets. Fig 4 and 5 show the chromatogram of volunteer before (blank) and after administration of 400 mg norfloxacin. The mean peak plasma concentration value, Cmax, in this study was about 900 ng/mL. The present method with LOQ of 30 ng/mL was sufficiently sensitive to analyze the plasma samples.
Table 2: Stability studies of norfloxacin.

<table>
<thead>
<tr>
<th>Stability study</th>
<th>Norfloxacin concentration (ng/mL)</th>
<th>120</th>
<th>Mean</th>
<th>SD</th>
<th>RSD</th>
<th>2000</th>
<th>Mean</th>
<th>SD</th>
<th>RSD</th>
<th>20000</th>
<th>Mean</th>
<th>SD</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze and thaw</td>
<td></td>
<td></td>
<td>98.17</td>
<td>1.36</td>
<td>1.39</td>
<td>90.32</td>
<td>2.43</td>
<td>2.69</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Short-term</td>
<td></td>
<td></td>
<td>95.16</td>
<td>5.62</td>
<td>5.90</td>
<td>98.69</td>
<td>2.41</td>
<td>2.44</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Long-term</td>
<td></td>
<td></td>
<td>98.79</td>
<td>5.84</td>
<td>5.92</td>
<td>96.07</td>
<td>1.93</td>
<td>2.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Post-preparative</td>
<td></td>
<td></td>
<td>105.83</td>
<td>10.57</td>
<td>9.99</td>
<td>101.81</td>
<td>2.77</td>
<td>2.72</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stock solution</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100.97</td>
<td>0.14</td>
<td>0.14</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± standard deviation, n = 3.

Fig. 3: Mean plasma concentration-time curve of norfloxacin of 18 volunteers after oral administration of 400 mg of norfloxacin of Norgan® and Norflo® tablets

Fig. 4: Chromatogram of plasma before drug administration

Fig. 5: Chromatogram of plasma 0.25 hr after drug administration.
CONCLUSION
A rapid, simple, selective and sensitive HPLC method for determination of norfloxacin in human plasma was successfully developed and applied to quantify norfloxacin concentration in bioequivalence study. The method showed good recovery, accuracy and precision. The simple sample preparation and short run time of 5 minutes were ideal for studies with large sample size.

ACKNOWLEDGEMENT
This research was fully supported by the grant with grant number 1001/PFARMASI/815071 and the authors would like to thank Institute of Postgraduate Study, Universiti Sains Malaysia, for providing fellowship.

REFERENCE