BIOEQUIVALENCE OF NORFLOXACIN BY HPLC-UV METHOD

MUHAMMAD SHER^{*,1}, MUHAMMAD AJAZ HUSSAIN¹, MHAMMAD HAMMAD MEHMOOD¹,
MUHAMMAD NAEEMUL HASSAN¹, SAJID BASHIR²

¹Department of Chemistry, University of Sargodha, Sargodha 40100, Pakistan

²Department of Pharmacy, University of Sargodha, Sargodha 40100, Pakistan

(Received: July 14, 2009 - Accepted: December 14, 2009)

ABSTRACT

A simple, rapid and convenient high performance liquid chromatographic method has been developed for the determination and bioequivalence of norfloxacin in tablets formulations by using ciprofloxacin as an internal standard. The chromatographic separation was achieved on a C18 column having an isocratic mixture of methanol, disodium hydrogen phosphate buffer and acetonitrile (30:30:40) at pH 3 adjusted with *o*-phosphoric acid. The eluents were detected at 280 nm and quantitation was achieved on the basis of peak height ratio of norfloxacin and internal standard. Limit of detection of the method developed was 15 ng mL⁻¹ with a linear range of 30 – 200 ng mL⁻¹. After single oral dose (400 mg) of two formulations (Noroxin, MSD and Ecoflaxin, Technovision Pharmaceutical) of norfloxacin administered to healthy volunteers using a randomize 2×2 crossover design, pharmacokinetics parameters (AUC₀₋₄, AUC_{0-∞}, C_{max}, t_{1/2}) were derived from the plasma concentrations curves for both formulations. Pharmacokinetic analysis of the data showed that the two formulations were bioequivalent, while no adverse reactions to drug were observed. In addition, the method evolved has been found suitable for the routine quality control tests.

Keywords: Norfloxacin, Bioequivalence, HPLC-UV

INTRODUCTION

Norfloxacin (1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1*H*-quinoline-3-carboxylic acid) is a broad spectrum synthetic fluoroquinolone antibiotic (Fig. 1). The addition of a fluorine atom at C-6 and a piperazine ring at C-7 has increased its effectiveness in relative to other fluoroquinolones. Norfloxacin solutions are photosensitive to daylight and to incandescent light^{1,2}.

Fig. 1. Structure of norfloxacin.

Norfloxacin is well tolerated and widely used against both gram-positive and gram-negative infections, including *Neisseria gonorrhoeae*, gentamycin-resistant *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and methicillin-resistant *Staphylococcus aureus*^{1,3,4}. It is mainly used in the treatment of urinary, respiratory and gastrointestinal tract infections³.

In fasting conditions, about 30–40% of an oral dose of norfloxacin is absorbed. Norfloxacin is excreted through biliary and renal excretions after metabolism. After an oral dose of 400 mg, plasma peak concentration (C_{max}) of drug is achieved within 3 h and plasma half-life⁵ is 5 h. Within 24 h, about 30% of dose is excreted unchanged in urine. It is reported that some metabolism occurs possibly in the liver⁶⁻¹⁰.

High performance liquid chromatography (HPLC) is the most commonly used technique for the determination of norfloxacin in biological fluids and in pharmaceutical dosage forms¹¹⁻¹⁴. A number of methods have been reported in the literature for the quantification of norfloxacin in human plasma and in animal tissues employing different extraction and detection strategies. Liquid-liquid extraction with ultraviolet detection¹⁵, and protein precipitation with UV¹⁶ or fluorescence detection¹⁷ are most commonly used techniques. Liquid chromatography coupled with mass spectrometry is also used for the determination of norfloxacin^{18,19}.

Bioavailability and pharmacokinetics of drugs allow manipulating their clinical, therapeutic and toxic effects. Additionally, these facilitate in monitoring the route of therapy. The difference in the bioavailability of various formulations of the same drug, having same strength and in the same dosage form

causes a special challenge to health care expert. Norfloxacin is available in the market in various dosage forms. The bioavailability and pharmacokinetics parameters can be greatly affected by the way of manufacturing and formulation of drug¹⁹.

The aim of this study was to assess the bioequivalence of a locally produced norfloxacin tablet brand (Ecoflaxin) and the original commercially available product (Noroxin) in healthy male Pakistani volunteers. As most preferable mode for the assays of different drugs in pharmaceutical industries is HPLC coupled with ultra-violet/visible (UV) detector instead of fluorescence detector. Therefore, present study is focused to develop a novel and simple HPLC-UV method for the determination of norfloxacin.

EXPERIMENTAL

Materials: All chemicals used for HPLC analysis were of analytical reagent grade obtained from E. Merck, Germany. Norfloxacin and ciprofloxacin (99.7% pure each) standards was gifted by Technovision Pharmaceutical, Islamabad, Pakistan.

Study participants: Twelve healthy humans (aged between 18 and 25 years, median age of 21 with 10 % ideal weight, mean 55 kg) were selected. The participants were educated about the type of study: the safety of the medicine and possible undesirable effects, etc., and consent was obtained. All the procedures followed were in accordance with the current revision of the Helsinki Declaration²⁰, and all the subjects used in this study gave their informed consent. The study was approved by the Ethics Committee, Medical College, University of Sargodha. The study was carried out at the affiliated hospital (District Head Quarters) of the University of Sargodha.

Study design: The participants were kept on fast at least 10 h (over night). They were stopped taking water 1 h before drug administration. A single dose of 400 mg of the test product and reference standard product was administered to the participants along with 240 mL of water. The participants were kept fasting for 5 h after administration of the drug. They were allowed to take water 1 h after administration of the drug during this fast. After that standard meals were served throughout the study. The washout phase between two treatments was at least 1 week. Three to five mL of venous blood samples were collected from each participant by using disposable syringes, canulas, and butterflies under aseptic conditions. Two participants were dropped out due to their personal reasons. Blood was collected from antecubital vein. Heparin (Leo, Denmark) was used as an anticoagulant. The blood samples were collected in centrifuge test tubes and were arranged in order on test tube racks and labeled accordingly with great accuracy. Blood samples were collected just before (blank) and after 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 36.0, 72.0, h of administration of the drug. The blood samples were centrifuged at 3000×g for 3 min. Plasma were separated by using micropipette with sucker and stored in refrigerator at 4°C in specially capped test tubes.

Standard preparation: The standard stock solutions of norfloxacin and ciprofloxacin were prepared by dissolving 100 mg of standard material in the

mobile phase (100 mL) separately. Ten (10) mL of this solution was diluted to 100 mL with mobile phase. The solution was filtered through 0.45- μ filter and degassed before use.

Plasma sample preparation: Plasma samples (250 μ L) were transferred to a 2 mL polypropylene vial to which internal standard (50 μ L, 4000 ng mL⁻¹) and 1 mL of acetic acid and acetonitrile was added. The samples were centrifuged at 3000 $\times$ g for 1 min. The vials were then frozen and the aqueous layer discarded. The organic phase was transferred to 2 mL glass vials and the solvent was evaporated to dryness at 40°C under a stream of nitrogen. The residue was redissolved in 250 μ L of mobile phase, of which 200 μ L was transferred into 250 μ L glass vials and placed in the autosampler for analysis. The injection volume was 20 μ L. Sample vials were wrapped in aluminium foil to protect norfloxacin from light exposure.

Instrumentation and conditions: The HPLC system comprised of G1311A quaternary pump, G1315B DAD variable wavelength UV detector and 1200 system controller (all from Agilent, Germany). The column used was Shim-Pak ODS 5 μ m (4.6 $\times$ 250 mm) while mobile phase used was a methanol, dihydrogen sodium phosphate buffer (pH 3.5, 0.05M) and acetonitrile (30:30:40) mixture at pH 3.5 adjusted with *o*-phosphoric acid. The flow rate, detection wave length, and injection volume were 1.0 mL min⁻¹, 280 nm and 20 μ L, respectively.

Method validation: Method validation was carried out in the plasma as per reported method²¹. The accuracy and precision were determined by use of quality control sample prepared by adding to the plasma in known amount (lying in the middle range of entire standard curve) of standard, from three concentrations representing the entire range of standard curve; one within 3 $\times$ times of the lower limit of quantitation (low quality control sample), one near the center (middle quality control) and one near the upper boundary of standard curve (high quality control). Measurements were made as ten replicates at each concentration and mean and coefficient of variance (CV) were thoroughly calculated. Linearity was calculated by preparing ten different concentrations of norfloxacin along with ciprofloxacin as internal standard in the plasma. 20 μ L of each concentration was injected.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated by preparing the solutions of norfloxacin and ciprofloxacin in the mobile phase as well as in plasma and were diluted to known concentrations to a final response equal to three times of the signal-to-noise ratio. The LOQ was taken as ten times of signal-to-noise ratio. The specificity of the method was determined by using six different plasma samples. The calibration curves of drugs in the mobile phase as well as in plasma were generated by using six concentrations. The quality control samples were used to accept or reject the run. The replicate measurements were made at three concentrations, one at lower limit of quantification, one in the mid range and another approaching high end of the range.

Specimen analysis: The plasma samples were analyzed by the HPLC method validated above, and concentration of norfloxacin was determined.

Bioequivalence (pharmacokinetic evaluation): Concentration-time curves were plotted and following parameters were determined: AUC_{0-t}, the area under the curve from time zero to time t; AUC_{0- ∞} , the area under the curve from time zero to time infinity using the formula $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$; $t_{1/2} = 0.693/k_e$, the half-life of the drug; C_{max} , the peak drug concentration; t_{max} , the time to peak drug concentration. $Cl = Dose / AUC_{0-\infty}$, Clearance. The area under curve was calculated from concentration-time curves by using linear trapezoidal method. The terminal rate constant, k_e , was determined by regression analysis of at least three data points in the terminal phase. The statistical analysis was performed by use of Statgraphics® 5.1. For the comparison of two sets of data F test was applied.

RESULTS AND DISCUSSION

Norfloxacin determination: The determination of norfloxacin was carried out by using the validated HPLC-UV method. Typical chromatograms showing the separation of norfloxacin and ciprofloxacin in the plasma have been shown in Fig. 2.

Fig. 2. Chromatogram showing norfloxacin (2.46) and ciprofloxacin (2.81).

The LOD was calculated from linear calibration curves by using standard deviation method. The LOD and LOQ values for norfloxacin were 15 and 30 ng mL⁻¹, respectively. Linearity studies have shown the linear range of drug ranges from 30 – 200 ng mL⁻¹. Linearity results are shown in Fig. 3.

Between days precision near limit of detection (LOD) in terms of coefficient of variation (CV) ranged from 0.2 to 0.9, and accuracy in terms of percent recovery was found to be greater than 99.57% for norfloxacin in plasma. All performance parameters clearly established the validity of the HPLC method for this study. The specificity and selectivity of the HPLC system were determined by a separate chromatographic analysis of the plasma samples. All results are summarized in Table 1. The method developed was found to possess better performance parameters as compared to the available methods in literature^{12,13}.

Table 1. Validation parameters of HPLC analysis of plasma.

Parameter	Norfloxacin (Means)
Precision (CV, within day/between days)	i) 0.2/0.5 at 15 ngmL ⁻¹ ii) 0.5/0.9 at 50 μ gmL ⁻¹ iii) 0.3/0.8 at 200 μ gmL ⁻¹
Accuracy (% recovery)	i) 99.57 at 15 ngmL ⁻¹ ii) 99.85 at 50 μ gmL ⁻¹ iii) 99.95at 200 μ gmL ⁻¹
LOD (ngmL ⁻¹)	15.0
Tailing factor, As	1.01
LOQ (ngmL ⁻¹)	30.0
Theoretical plates, N	10542
Resolution, Rs ^a	1.3
Capacity factor, k	2.2

^aResolution between the adjacent peaks.

Fig. 3. Calibration curve of Norfloxacin in plasma.

Bioequivalence (pharmacokinetic evaluation): For bioequivalence study the pharmacokinetics parameters of both formulations were calculated. The pharmacokinetics data following the single oral administration of 400 mg norfloxacin (both formulations) are given in Table 2.

Table 2. Pharmacokinetic data after a single oral dose of 400 mg norfloxacin (Noroxin, MSD & Ecoflaxin, Technovision pharma)

Parameter	Noroxin ^a	Ecoflaxin ^a
$t_{\max}$, h	1.75 (0.35)	1.75 (0.30)
$C_{\max}$, $\mu\text{g mL}^{-1}$	8.9 (0.08)	8.78 (0.07)
$t_{1/2}$, h	3.76 (0.4)	3.55 (0.5)
$\text{AUC}_{0-\infty\text{h}}$, h. $\mu\text{g mL}^{-1}$	5.86 (0.07)	5.07 (0.06)
Clearance ^b , Lh^{-1}	70.20 (3)	69.90 (3)

^a Values are means ($\pm$ standard deviations)

^b Total body clearance for extra-vascular administration

The plasma concentration-time curves are shown in Fig. 4 for Noroxin and Ecoflaxin. All pharmacokinetics parameters; $t_{\max}$, h; $C_{\max}$, mg mL^{-1} ; $t_{1/2}$, h; $\text{AUC}_{0-\infty\text{h}}$, h. mg mL^{-1} and Cl of both formulation were found to be closely related. It was found that $F < F_{\text{critical}}$, which clearly indicates that there is no significant difference between the two sets of data. The results are given in Table 3.

Fig. 4. Mean plasma norfloxacin concentration versus time curve after a single 400 mg dose of Noroxin (◆) and Ecoflaxin (▲).

Table 3. Result of F-test applied on Pharmacokinetic data of norfloxacin (Noroxin, MSD & Ecoflaxin, Technovision pharma)

Parameter	Noroxin	Ecoflaxin	F-test result
$t_{\max}$, h	1.75	1.75	0.409
$C_{\max}$, $\mu\text{g mL}^{-1}$	8.9	8.78	0.930
$t_{1/2}$, h	3.76	3.55	0.769
$\text{AUC}_{0-\infty\text{h}}$, h. $\mu\text{g mL}^{-1}$	5.86	5.07	0.966
Clearance ^b , Lh^{-1}	70.20	69.90	0.864

In conclusion, we described here the development of a new, selective, precise and accurate method for the quantification of norfloxacin in human plasma using HPLC with UV detection and liquid-liquid sample extraction which was applied to a bioequivalence study. The method reported here uses a simple and effective extraction technique with good and reproducible recovery and a limit of quantification of 15 ng mL^{-1} . The developed method is suitable for pharmacokinetic studies of norfloxacin as well. The study was able to demonstrate bioequivalence between the two formulations with a 90% confidence interval. Ahead of the clinical observations carried through during and after the study no adverse effect was observed, showing a good tolerability to the both formulations.

ACKNOWLEDGMENTS

We thank Mr. Muhammad Yasir, Department of Pharmacy, University of Sargodha for his thoughtful assistance for the analysis of pharmacological data.

In addition, we express our appreciation to Surgeon Dr. Abdul Latif and Dr. Mazhar for their support in conducting the study at DHQ Hospital, Sargodha. We also thank the study subjects for their participation. None of the authors has a conflict of interest.

REFERENCES

1. M. Cordoba-Borrego, M. Cordoba-Diaz, D. Cordoba-Diaz, J.Pharm. Biomed. Anal. **18** (6), 919, (1999).
2. A. Espinosa-Mansilla, A. M. Delapena, D. G. Gomez, F. S. Lopez, Talanta, **68** (4), 1215, (2006).
3. R. N. Rao, V. Nagaraju, J. Pharm. Biomed. Anal. **34** (5), 1049, (2004).
4. J. Lim, B. Park, H. Yun, J. Chromatogr. B. **772**, 185, (2002).
5. R. Farinotti, G. Peytavin, Antibiotiques. In: Labaune, J. (Ed.). Propriétés pharmacocinetiques des médicaments. 1.ed. Paris: Masson, 144, (1999).
6. C. Carson, K. G. Naber, Drugs, **64**, 1359, (2004).
7. S. C. Sweetman (ed): Martindale, The complete drug reference, Ed 34. Pharmaceutical press, London, 239, (2004).
8. P.D.R Generics, Ed 3. Medical Economics, Montvale, (1997).
9. J. L. Vilchez, O. Ballesteros, J. Taoufiki, G. Sanchez-Palencia, A. Navalón, Anal. Chim. Acta, **444**(2), 279, (2001).
10. H. J. Mascher, C. Kikuta, J. Chromatogr. A, **812**(1-2), 381, (1998).
11. G. Carlucci, J. Chromatogr. A. **812**(1-2), 343, (1998).
12. C. G. B. Danilo, M. G. Talita, L. B. Leila, E. M. Carlos-de Sousa, R. P. Luis-de Abreu, O. Eduardo-de Jesus, P. Davi-de Santana, Brazilian journal of pharmaceutical sciences, **43**(2), 231, (2007).
13. A. H. Nada, M. A. Sharaf, Z. A. El Gholmy, N. M. Khalafallah, Med. Princ. Pract. **16**, 426, (2007).
14. T. Galaon, S. Udrescu, I. Sora, V. David, A. Medvedovici, Biomed. Chromatogr. **21**, 40, (2007).
15. S. C. Wallis, B. G. Charles, L. R. Gahan, J. Chromatogr. B, **674**(2), 306, (1995).
16. V. F. Samanidou, C. E. Demetrious, I. N. Papadoyannis, Anal. Bioanal. Chem. **375**(5), 623, (2003).
17. M. Well, K. G. Naber, M. Kinzig-Schippers, F. Sorgel, Int. J. Antimicrob. Agents, **10**(1), 31, (1998).
18. B. Toussaint, G. Bording, A. Janosi, A. R. Podriguez, J. Chromatogr. A. **976**(1-2), 195, (2002).
19. O. Ballesteros, I. Toro, V. Sanz-Nebot, A. Navalona, J. L. Vilchez, J. Barbosa, J. Chromatogr. B. **798**(1), 137, (2003).
20. Eckenwiler, L., F. Dafna, E. Carolyn, S. Toby, "The Declaration of Helsinki through a feminist lens" Int. J. Feminist Approaches Bioethics **1**(1), 161, (2008).
21. P. Vinod, P., K. Kamal, W.A. John, M. Howard, D. James, J. Iain, N. Rabindra, L. Mark, T. Alfred, C. Viswanathan, and T. Avraham.. Pharm. Res. **17**, 1551, (2000).