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Simple and rapid LC-method for determination of moxifloxacin in human plasma: A bioequivalence study in healthy volunteers

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ABSTRACT

A simple isocratic reversed-phase high performance liquid chromatographic method with ultraviolet detection was developed and validated for determination of moxifloxacin in human plasma using gatifloxacin as an internal standard. The developed method was validated after optimization of various chromatographic conditions and other experimental parameters. Samples were separated using Inertsil C_{18} (250 x 4.6 mm, 5 µm) analytical column. The mobile phase, methanol/30mM KH_2PO_4 buffer (pH 2.5) (40:60, v/v) operated at 35°C column oven temperature was pumped at a flow rate of 1.0 mL/min. The column eluents were monitored at 300 nm. The present method demonstrated acceptable values for selectivity, linearity within the expected concentration range (0.1-5 µg/mL; $r^2 = 0.9999$ for moxifloxacin. The method was efficiently applied to support a bioequivalence study of 400 mg moxifloxacin tablets in 24 healthy subjects.

KEYWORDS

Bioequivalence;
Gatifloxacin;
HPLC-UV;
Moxifloxacin;
Plasma;
Validation.

INTRODUCTION

Moxifloxacin hydrochloride (MOX), is 1-Cyclopropyl-6-fluoro-8-methoxy-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-*b*]pyridin-6-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid hydrochloride[1], Figure 1. It is a fluoroquinolone antibacterial agent with a broad spectrum activity, encompassing gram-negative and gram-positive bacteria[2].

Different methods for the quantification of MOX in biological fluids were developed. MOX was determined in tablets, human urine and serum by spectrofluorimetry[3], square-wave adsorptive voltammetry[4], capillary electrophoresis[5]. MOX was assayed in serum using HPLC and fluorescence detection[6]. MOX was determined in growth media by HPLC and fluorescence detection[7]. MOX was determined in plasma and lung tissue by HPLC-UV detection using solid phase extraction[8]. MOX was determined in different biological fluids using HPLC-MS methods[9-11].

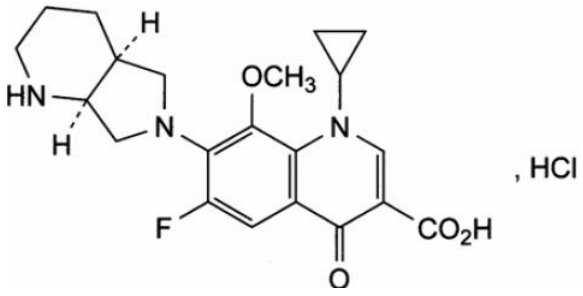

Figure 1 : Structure of moxifloxacin hydrochloride

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Difficulties may be met in the analysis of fluoroquinolones both in extraction and in the chromatographic steps. These molecules are weak heterocyclic amino acids with two reactive sites: an amino group which can be protonated and a carboxyl group which can lose a proton[12]. Due to this amphoteric character of fluoroquinolones, they may exist in cationic, neutral, zwitter ionic and anionic forms.

The aim of this investigation was to develop and validate a simple, single and rapid HPLC method with UV detection for the quantification of moxifloxacin in human plasma. In order to allow high throughput analysis, required for a pharmacokinetic and bioequivalence study, this method must involve minimal sample pretreatment and a short analysis time. A judicious use of protein precipitation and the pursuit for sample extract compatibility with an optimized chromatographic separation constitutes the basis of the resulting robust analysis procedure.

EXPERIMENTAL

Reagents and chemicals

Moxifloxacin hydrochloride (MOX) pure powder was kindly supplied by Bayer Schering Pharma AG, (Leverkusen, Germany). Its purity was found to be 100.45 ± 0.840 (n=6) according to the BP HPLC method[1]. Gatifloxacin (GTF) pure powder was purchased from ZHECHEM, (Hangzhou, China). Its purity was found to be 99.32 ± 0.762 (n=6) according to a reported method[13]. Acetonitrile and methanol were HPLC grade (Sigma Aldrich, Germany). Potassium dihydrogen phosphate was purchased (Scharlau, Spain). Double distilled water (Aquatron, U.K). Blank plasma; was obtained from National Institute of Urology and Nephrology (Egypt) and was stored at -80 °C.

Pharmaceutical formulation

Actimoxiflox 400 mg tablets, (test product), Batch No.: 12004, manufactured by International Drug Agency for Pharmaceutical Industry for Mira International for Pharmaceuticals and Chemicals, Egypt, (Exp. date: 01/2014).

Avalox® 400 mg tablets, (reference product), Batch No.: BXG0DH1, manufactured by Bayer Schering Pharma AG, Leverkusen, Germany, (Exp. date: 07/2014).

Instrumentation

Quantitative analysis was performed on Agilent LC system, quaternary pump: G1311A, degasser: G1322A, autosampler: G13329A, UV- detector: G1315D, (Böblingen, Germany). Chromatographic separation of analytes was carried out on an Inertsil C_{18} (250 x 4.6 mm, 5 µm) analytical column using methanol: 30mM potassium dihydrogen phosphate, pH 2.5 adjusted with phosphoric acid (40:60, v/v) as the mobile phase at a flow rate of 1 mL/min., isocratically. The column was maintained at 35°C. The injection volume was 20 µL and UV detection was done at 300 nm.

Calibrators and quality control samples

The standard stock solution of MOX (1000 µg/mL) was prepared by dissolving 109 mg of moxifloxacin hydrochloride, equivalent to 100 mg free moxifloxacin into 100 mL volumetric flask using distilled water. Working solutions of 5, 10, 25, 50, 100, 150, 200 and 250 µg/mL were prepared in distilled water. Calibration standards and quality control (QC) samples were prepared by spiking 2% of total plasma volume, 20µL of each working solution to 980 µL blank plasma. Calibration standards were made at the following plasma concentrations: 0.1, 0.2, 0.5, 1, 2, 3, 4 and 5 µg/mL.

The QC samples were prepared at three concentration levels; 0.3 µg/mL (LQC, low quality control), 1.5 µg/mL (MQC, medium quality control), 2.5 µg/mL (HQC, high quality control). Separate stock solution (600 µg/mL) of the internal standard, gatifloxacin, was prepared by dissolving 60 mg of gatifloxacin reference standard in 100 mL of acetonitrile. Precipitating solution was prepared by taking 2 mL of gatifloxacin stock solution into 200 mL volumetric flask and the volume was completed using acetonitrile to reach a concentration of 6 µg/mL of gatifloxacin. Standard stock and working solutions used for spiking were stored at 5 °C, while calibration standards and QC samples in plasma were kept at -80 °C until use.

Sample preparation

A volume of 2 mL of the precipitating solution containing the IS was added to 1 mL plasma containing MOX. Vortex was done for 1 minute followed by centrifugation at 4000 r.p.m. for 15 minutes. A volume of

20 µL from the clear supernatant layer was injected. The peaks were detected by diode array detector at 300 nm and were interpreted in the form of reported peak areas. Calibration curve was obtained by plotting the relative peak areas, MOX/IS, versus the corresponding concentration of MOX. Concentrations of MOX in unknown samples were calculated by referring to the repeated calibration curve.

Method validation

The proposed analytical method was validated according to international guidelines with emphasis on selectivity, linearity within the expected concentration range, recovery, precision (repeatability and intermediate precision), sensitivity, stability of solutions, and robustness.

Selectivity

The selectivity of the method towards endogenous plasma matrix components was verified in six batches of blank human plasma. In addition, interference owing to some commonly used medications by human volunteers was also checked. These included paracetamol, chlorpheniramine maleate, diclofenac, caffeine and ibuprofen. Their working solutions (100 µg/mL) were prepared in the mobile phase and 20 µL was injected to check for any possible interference at the retention time of MOX and IS.

Linearity

The linearity of the method was assessed by analysis of six linearity sets. The area ratio response for analyte/IS obtained was plotted against MOX plasma concentration. The lowest standard on the calibration curve (LLOQ) was accepted as its response was at least 10 times more than that of drug-free (blank) extracted plasma.

Extraction recovery and matrix effect

The extraction recovery for MOX and IS was calculated by comparing the mean area response of samples spiked before extraction to that of extracts with post-spiked samples (spiked after extraction) at three QC levels. The absolute matrix effect was estimated by comparing the mean area response of post-spiked samples with mean area response of solutions prepared in mobile phase (neat standards). Relative matrix effect was assessed from the precision values of the slopes of the calibration curves prepared from six different plasma lots.

Intra- and inter-day precision and accuracy

Intra-day precision and accuracy was determined by analyzing six replicates of QC samples along with calibration curve standards on a single day. The inter-day accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive days. The precision at each concentration level from the nominal concentration was expected to be not greater than 15% and the accuracy to be within ±15% as per US FDA guidelines[14], except for the LLOQ, where it can be 80-120% of the nominal concentration. Re-injection reproducibility was checked by re-injecting one entire validation batch.

Sensitivity

The sensitivity of the method was evaluated by determining the limit of detection (LOD) and LLOQ using the signal-to-noise ratio (S/N) approach at the values of 3 and 10, respectively.

Robustness

To determine the robustness of the developed method, effect of small deliberate variations in system parameters like the organic component of the mobile phase (±2%), the mobile phase flow rate (±0.1 mL/min.), the column oven temperature (±5 °C), and the detection wavelength (±1 nm) was studied.

Stability

Stability tests were conducted for stock solutions of MOX and IS for short-term and long-term stability at 25 and 5°C, respectively. The acceptance criterion was ±10.0% deviation from the nominal value. All stability results for spiked plasma samples were evaluated by measuring the area response ratio (analyte/IS) of stability samples against freshly prepared comparison standards at the three QC levels.

Three cycles of freeze-thaw and long-term stability of the analyte in plasma (at -80 °C for 30 days) were also studied at these QCs levels. Whole blood stability was determined to ascertain any enzymatic degrada-

tion by spiking blood samples with analyte at the three QC levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly prepared quality control samples was within ±15.0%.

Ruggedness and dilution integrity

Method ruggedness was estimated on two precision and accuracy batches. The first batch was studied on two Inertsil C_{18} (250 x 4.6mm, 5 µm) columns with different batch numbers, while the second batch was analyzed by different analysts who were not part of method validation. Dilution integrity was determined by diluting the stock solution prepared as spiked standard at 10 µg/mL for MOX in the blank screened human plasma. The results obtained for 1:5 and 1:10 dilution were determined against freshly prepared calibration curve standards.

System suitability, system performance and auto-sampler carryover

The system suitability test was conducted by injecting six consecutive injections using an aqueous standard solution of MOX (at upper limit of quantitation) and IS at the start of each batch during method validation.

System performance was studied by injecting one extracted blank (without analyte and IS) and one extracted LLOQ sample with IS at the beginning of each analytical batch. The carryover effect of the auto-sampler was evaluated by sequentially injecting system suitability samples, blank, zero, calibrators starting from the lowest to the highest level, and then blank solvent at the start of each batch.

Pharmacokinetic/bioequivalence study and statistical analysis

The purpose of the study was to investigate the bioequivalence of one tablet of Actimoxiflox 400 mg tablets, Mira International for Pharmaceuticals and Chemicals, Egypt (test product) and one tablet of Avalox® 400 mg tablets, Bayer Schering Pharma AG, Leverkusen, Germany (reference product) after oral administration to healthy adult volunteers under fasting conditions. The design of the study was an open label, balanced, randomized, two-treatment, two-period, two-sequence, crossover, single-dose bioequivalence study in 24 healthy adult Egyptian subjects under fast conditions. The primary end point or target variables of the study were C_{max}, $AUC_{0\text{-}36\ hrs}$ and $AUC_{0\text{-}inf}$, which were analyzed using the confidence interval approach. The secondary end points of the study included $AUC_{0\text{-}36hrs}$, $AUC_{0\text{-}inf}$, T_{max}, K_{el} and $t_{1/2}$.

The concerned subjects were informed about the objectives and possible risks involved in the study and a written consent was obtained. The study was conducted as per International Conference on Harmonization and US-FDA guidelines[14]. The subjects were orally administered a single dose of test and reference formulations with 240mL of water after a recommended washout period of one week. Blood samples were collected at 0.00 (pre-dose), 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 30.0 and 36.0 hrs after oral administration of the dose for test and reference formulations. Blood samples were collected through a cannula inserted into the subject's forearm vein into glass tubes containing 0.1 mL of heparin as anticoagulant, and centrifuged at 4000 rpm for 10 minutes. After centrifugation, plasma samples were transferred directly into 5-mL plastic tubes. These samples were immediately stored at the study site in a freezer (at -80°C). During the study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic parameters of MOX were estimated by non-compartmental analysis using in-house validated excel software.

The 90% confidence interval for the difference of means between the two formulations least square means was calculated for the target variable using log-transformed data. Similarly, power and ratio analysis was performed on the log transformed data. The terminal end points for the elimination rate constant were automatically selected using the software using the best fit model. To determine whether the test and reference formulations were pharmacokinetically equivalent, C_{max}, $AUC_{0\text{-}36\ hrs}$ and $AUC_{0\text{–}inf}$ and their ratios (test/reference) using log transformed data were assessed. The drug formulations were considered pharmacokinetically equivalent if the difference between the compared parameters was statistically non significant (p e" 0.05) and the 90% confidence intervals for these parameters were within 0.8-1.25.

RESULTS AND DISCUSSION

Optimization of sample preparation and chromatographic conditions

Separation of ionizable analytes such as acids and bases in terms of column efficiency, selectivity and retention depends on the pH of the mobile phase and the chromatographic conditions used. Assay of drugs in human plasma is an important issue for bioequivalence studies. A simple procedure based on protein precipitation was applied for determination of MOX in human plasma. Two mL of the precipitating solution (600 µg/mL gatifloxacin in acetoniltrile) were added to 1 ml plasma, vortexed for one minute, followed by centrifugation (at 4000 rpm) for 10 minutes. A volume of 20 µL from the supernatant clear layer of the prepared sample was injected into the column. The peaks were detected by diode array detector at 300 nm and were interpreted in the form of reported peak areas. Concentrations of moxifloxacin in unknown samples were calculated by referring to the prepared calibration curve. To optimize the proposed HPLC-UV method, the effects of several chromatographic parameters were investigated. These included the type of organic modifier, buffer, the concentration and pH of the buffer, and organic modifier-buffer ratio. These parameters were optimized based on the peak shape, peak intensity/area, peak resolution and retention time for the analytes on Agilent 1200 HPLC, Inertsil C_{18} (250 x 4.6 mm, 5 µm) column. Figure 2 shows the HPL Chromatogram of blank human plasma, while Figure 3 shows the HPL Chromatogram of blank plasma spiked with the internal standard. Figure 4 shows a good resolution of MOX and IS from plasma peaks of a volunteer.

Method validation

Selectivity

The method was selective for determination of MOX in human plasma. The target peak was well resolved from the internal standard, from other peaks of extraneous and endogenous substances in spiked plasma samples. The blank plasma sample; and the plasma sample spiked with the internal standard and a representative chromatogram of a volunteer are shown in Figures 2, 3 and 4, in order.

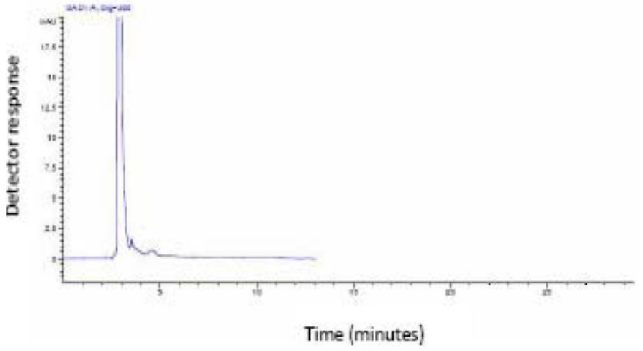

Figure 2 : HPL chromatogram of blank human plasma

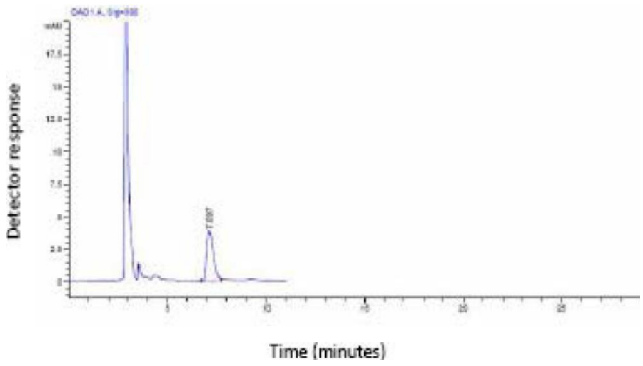

Figure 3 : HPL chromatogram of blank human plasma and gatefloxacin (IS)

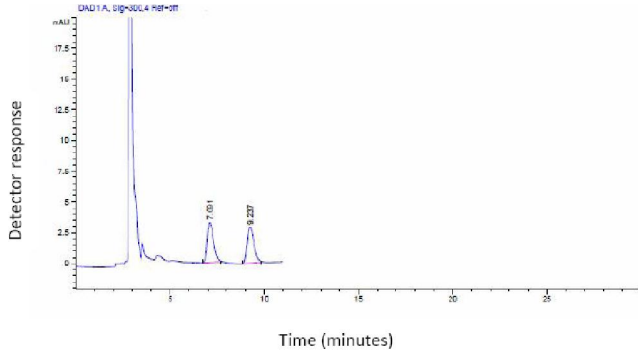

Figure 4 : Representative HPL chromatogram of a volunteer after 1 hr

Linearity

The proposed method shows a good linearity within the studied concentration range of 0.1-5.0 µg/mL. The regression equation, calibration range and the correlation co-efficient are summarized in TABLE 1.

Sensitivity

The LOD and LLOQ for MOX are also given in TABLE 1, showing that the proposed method is sensitive and that it can be used to determine the concentration of MOX in the plasma even after 36 hrs of single 400 mg oral dose.

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TABLE 1 : Calibration range, linearity, and sensitivity of the proposed method for determination of moxifloxacin hydrochloride by the proposed method

Parameter	Moxifloxacin
Range (µg/mL)	0.10 -5.00
Linearity	
Mean regression equation	y=0.399 x–0.003
Correlation co-efficient	0.9999
Sensitivity	
Limit of detection, LOD µg/mL	0.025
Lowe limit of quantification, LOQ µg/mL	0.100

y is the response ratio and x is the concentration (µg/mL)

TABLE 2 : Recovery and precision for determination of moxifloxacin hydrochloride by the proposed method

Parameter	Mean ± SD; %RSD
Recovery	
Spiked concentration level 1[a]	[d] 97.44 ± 2.69; 2.76
Spiked concentration level 2[a]	[d] 98.39 ± 1.57; 1.59
Spiked concentration level 3[a]	[d] 99.37 ± 0.28; 0.28
Precision	
Repeatability	
Injection repeatability[b]	[e] 9.14 ± 0.05; 0.55
Analysis repeatability[b]	[d] 99.25 ± 0.23; 0.23
Intermediate precision	
Intra-day	
Spiked concentration level 1[c]	[f] 0.315 ± 0.005; 1.587
Spiked concentration level 2[c]	[f] 1.461 ± 0.095; 6.502
Spiked concentration level 3[c]	[f] 2.427 ± 0.032; 1.318
Inter-day	
Spiked concentration level 1[c]	[f] 0.292 ± 0.021; 7.192
Spiked concentration level 2[c]	[f] 1.438 ± 0.091; 6.328
Spiked concentration level 3[c]	[f] 2.477 ± 0.035; 1.413
Analyst 2	
Spiked concentration level 1[c]	[f] 0.308 ± 0.011; 3.571
Spiked concentration level 2[c]	[f] 1.482 ± 0.102; 6.883
Spiked concentration level 3[c]	[f] 2.433 ± 0.095; 3.905

Spiked concentration level 1= 0.3 µg/mL; spiked concentration level 2= 1.5 µg/mL; spiked concentration level 3= 2.5 µg/Ml; [a] n=6; [b] n= 10; [c] n=5 (where n is the number of samples); [d] Recovery (%); [e] Retention time (min.); [f] Quantity recovered (µg/mL).

Recovery

Results of the recovery studies with the optimized procedure are summarized in TABLE 2, showing that the recovery of MOX was more than 95% at all the three nominal concentration levels for plasma samples.

Precision

Results of the repeatability (injection and analysis) and intermediate precision (intra- and inter-days reproducibility) are also summarized in TABLE 2, showing complete agreement among the repeated injections (both retention times and peak areas), repeated analyses, and intra- and inter-days studies.

Robustness

Results of slight changes in various system parameters like the organic component of the mobile phase, the mobile phase flow rate, the column oven temperature, and the detection wavelength indicated that the method was robust as its performance was negligibly affected by minor changes in these parameters.

Stability

Short-term stability study indicated that spiked plasma samples remained stable for at least 24 hrs even at room temperature (30 °C). On the other hand, standard solution of MOX remained stable for at least 1 week when kept frozen. Results of stability of MOX in human plasma samples are shown in TABLE 3.

Applicability of the method

There was no peaks of interfering with MOX and IS at their retention times in the blank and spiked plasma samples. Potential interferences by common drugs which are administered concurrently with MOX were tested and found not to interfere with MOX and IS retention times. The developed method was a part of an extensive pharmacokinetic and bioequivalence study in healthy human volunteers. Initially, the method was applied for studying the pharmacokinetics of MOX in healthy adult volunteers.

The method was applied for a bioequivalence study to investigate the bioequivalence of one tablet of Actimoxiflox 400 mg tablets, Mira International for

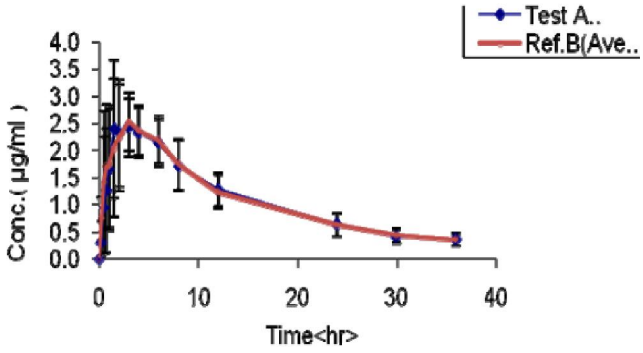

Figure 5 : Mean plasma concentration time curve (µg/mL) of moxifloxacin following administration of one tablet Actimoxiflox 400 mg (Generic A as a test product) and one tablet of Avalox® 400 mg (as a reference product) to 24 Volunteers

TABLE 3 : Stability of moxifloxacin hydrochloride in matrix by the proposed method

Parameter	Mean [b] ± SD; %RSD
Short term stability of analyte in matrix at room temperature	
Spiked concentration level 1[a]	94.67 ± 2.14; 2.26
Spiked concentration level 2[a]	92.12 ± 6.25; 6.78
Spiked concentration level 3[a]	99.32 ± 0.31; 0.31
Post-preparative stability at 4°C	
Spiked concentration level 1[a]	98.88 ± 1.86; 1.88
Spiked concentration level 2[a]	96.58 ± 1.13; 1.17
Spiked concentration level 3[a]	97.29 ± 0.50; 0.51
Long term stability of analyte in matrix at -80°C	
Spiked concentration level 1[a]	99.36 ± 1.76; 1.77
Spiked concentration level 2[a]	98.59 ± 1.19; 1.21
Spiked concentration level 3[a]	98.61 ± 1.74; 1.76
Freeze and thaw stability	
Spiked concentration level 1[a]	99.76 ± 1.93; 1.93
Spiked concentration level 2[a]	90.21 ± 4.51; 4.90
Spiked concentration level 3[a]	96.63 ± 2.36; 2.44

Spiked concentration level 1= 0.3 µg/mL; spiked concentration level 2= 1.5 µg/mL; spiked concentration level 3= 2.5 µg/mL; [a] n=6; [b] Recovery (%).

TABLE 4 : Pharmacokinetic parameters of moxifloxacin following oral administration of one tablet of Actimoxiflox 400 mg (test product) and one tablet of Avalox® 400 mg (reference product)

Parameter	Test	Reference
C_{max} (µg/mL)		
Mean	3.04 ± 0.74	2.98 ± 0.69
Range	1.99-4.67	1.57-4.60
T_{max} (hr)		
Mean	2	2
Range	0.5-6.0	0.5-8.0
$AUC_{0\text{-}36\ hrs}$ (µg r/mL)		
Mean	37.09 ± 7.15	37.44 ± 8.37
Range	23.10-47.53	18.43-49.25
$AUC_{0\text{-}inf}$ (µg hr/mL)		
Mean	43.61 ± 8.88	44.64 ± 10.42
Range	28.55-58.99	25.04-64.06
$k(hr^{-1})$		
Mean	0.0622 ± 0.023	0.0558 ± 0.010
Range	0.04-0.15	0.04-0.07
$t_{1/2}$(hr)		
Mean	12.14 ± 3.06	12.81 ± 2.35
Range	4.70-17.31	9.80-17.19

Pharmaceuticals and Chemicals (test product) and one tablet of Avalox® 400 mg tablets, Bayer Schering Pharma AG, Leverkusen, Germany (reference product) after oral administration to healthy adult volunteers under fasting condition, results of pharmacokinetic parameters are depicted in TABLE 4. The mean plasma concentration time curve (µg/mL) of moxifloxacin following administration of one tablet of Actimoxiflox 400 mg (test product) and one tablet of Avalox® (reference product) to 24 volunteers is shown in Figure 5.

CONCLUSION

The HPLC-UV method for the quantitation of MOX in human plasma was developed and fully validated as per US-FDA guidelines. The method offers several advantages over reported procedures, in terms of sensitivity, lower sample requirements, relatively simple sample preparation and overall short analysis time. The efficiency of protein precipitation and a chromatographic run time of 12 minutes per sample make it a rapid procedure in high-throughput bio-analysis. Absence of matrix interference is effectively shown by post-column infusion and by the precision values for the calculated slopes of calibration curves. The method was successfully used in the analysis of about 1200 samples in a clinical setting. Additionally, the reproducibility of the method is shown by reanalysis of 48 subject samples.

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