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Optimization and validation of the direct HPLC method for the determination of moxifloxacin in plasma

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Abstract

Moxifloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo-[3,4-*b*]pyridin-6-yl]-4-oxo-3-quinolinecarboxylic acid hydrochloride) is new, fourth generation fluoroquinolone with broaden spectrum of antibacterial activity. In the present work simple and rapid RP-HPLC method for the direct determination of moxifloxacin in human plasma is described. Separation of moxifloxacin from plasma components was achieved on Supelco LC-Hisep shielded hydrophobic phase column. The mobile phase consisted of acetonitrile and 0.25 mol/dm^3 Na_3PO_4 (pH 3) in a volume percent ratio (5:95, v/v) and was delivered at a rate of 1 mL/min. Fluorescence detection was employed with excitation at 290 nm and emission at 500 nm. Ofloxacin was used as internal standard and sodium dodecylsulfate solution was used as a displacing agent. Sample preparation was simplified and involved only addition of displacing agent and internal standard and dilution with water. The separation conditions were optimized by the response surface method in two factor space, i.e. the dependence of the retention time on volume percent of acetonitrile and on pH of aqueous phase was optimized. The method was fully validated and validation parameters were: linearity range 3–1300 μg/L; correlation coefficient, 0.99986; mean recovery, 92.5%; limit of quantification, 3.0 μg/L and limit of detection, 1.0 μg/L. Method was applied for the determination of moxifloxacin in human plasma after single or repeated oral doses of 400 mg Avelox® tablets. The proposed method proved to be rapid and accurate and can be successfully used in pharmacokinetic studies and routine clinical practice.

Keywords: Moxifloxacin; RP-HPLC; Determination; Plasma

1. Introduction

Moxifloxacin (1-cyclopropyl-7-(*S*,*S*)-2,8-diazabicyclo(4.3.0)-non-8-yl-6-fluoro-8-methoxy-1,4-dihydro-4-oxo-3-quinoline carboxylic acid hydrochloride), Scheme 1, is a new fourth generation 8-methoxy fluoroquinolone developed primarily for the treatment of community acquired pneumonia and upper respiratory tract infections. It is active not only against Gram-negative pathogens but also against Gram-positive cocci aerobic intracellular bacteria, atypical organisms and anaerobic bacteria [1]. Moxifloxacin is readily absorbed from gastrointestinal (gi) tract. It is 42% protein bound in plasma and penetrates easily into target tissues and fluids. It is mainly metabolized to the N-sulfate and acylglucuronide. The pharmacokinetics of moxifloxacin were found to be linear within a wide range of single oral and parentheral doses from 50 to 800 mg. Maximum concentration in plasma is reached after 0.5–4 h (0.29–4.73 mg/L) [2].

There is a need for pharmacokinetic and toxikinetic studies of moxifloxacin in human plasma. Thus, in this paper a direct plasma injection method for the determination of moxifloxacin is reported using restricted access media column (RAM). These columns are so constructed that spiked plasma can be directly injected onto the column without any prior extraction of the drug. Direct injection of untreated plasma samples onto a conventional reversed phase columns causes clogging and deterioration of the column. The RAM column used in the present paper is a shielded hydrophobic phase column (Hisep). The stationary phase of Hisep column is formed of hydrophilic network of bonded polyethylene oxide embedded with hydrophobic phenyl groups. Hydrophobic bonded phase works like a partitioning phase while hydrophilic polymer because of its pore

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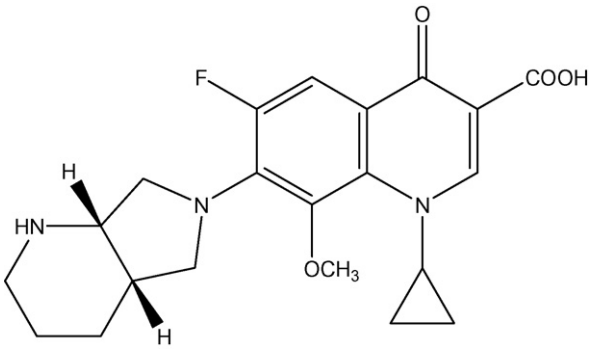

Scheme 1. Chemical structure of moxifloxacin.

size, excludes large polymers such as proteins, from interacting with the partitioning phase. When serum or plasma is injected onto a Hisep column the plasma proteins are size excluded by the outer hydrophilic shielding phase and flushed off with the solvent front, while small analytes, such as drugs, penetrate the water solvated interface of the hydrophilic network to interact with the hydrophobic regions (partitioning phase). The advantages of direct injection HPLC involve simplified sample preparation, shorter analysis time and good recovery of analytes. On the other hand this method suffers from the lack of sensitivity for analyzing low blood levels of some drugs [3,4].

Widespread use of moxifloxacin in clinical practice accentuates the need for rapid and reliable method of its determination in biofluids. However, not many methods have been developed for moxifloxacin determination in biological fluids and none of so far reported refer to the use of Hisep column. Stass and Dalhoff [5,6] have reported a method for moxifloxacin determination in human body fluids by high performance liquid chromatography with fluorescence detection (λ_{ex} = 296 nm, λ_{em} = 504 nm) using on column focusing. Separation was achieved on Nucleosil100 C_{18} column with a mobile phase consisting of tetrabuthylammonium sulphate and acetonitrile with pH 3.01 adjusted with NaH_2PO_4. Linear concentration range was from 5 to 1500 μg/L with limit of detection (LOD) 2.5 μg/L. Lemoine et al. [7] determined moxifloxacin in plasma and lung tissue by HPLC with UV detection (λ = 296 nm) using automated extraction method with a polymeric cartridge. Mobile phase consisted of acetonitrile and 10 mmol/L KH_2PO_4 (18:82, v/v) at pH 4.0. Linear range was 0.025–3.2 μg/mL and LOD = 0.0065 μg/mL. Ocaña et al. [8] determined moxifloxacin in tablets, urine and plasma by spectrofluorimetry using micellar medium (8 mmol/L sodium dodecylsulfate, SDS) with λ_{ex} = 294 nm and λ_{em} = 503 nm. Limit of detection was 15 ng/mL. Möller et al. [9] used capillary electrophoresis for the determination of moxifloxacin in very small samples of plasma. Laser induced fluorescence detection was employed at excitation wavelength 325 nm and emission at 520 nm. Dynamical range of 2.5–5000 μg/L and LOD = 0.5 μg/L compare well with standard HPLC method. Vishwanathan et al. [10] described the method based on LC/ESI–MS/MS for the quantitation of moxifloxacin in human plasma after previous solid phase extraction. Linear range was 1–1000 ng/mL and LOD = 50 pg/mL. Ba et al. [11] developed a direct injection HPLC method, using Supelcosil ABZ column and fluorescence detection, to determine moxifloxacin in Mueller–Hinton broth. Column switching technique was employed. Quantitation limit was 0.05 μg/mL. Erk [12] has reported the determination of moxifloxacin in dosage forms and human plasma by cyclic, differential pulse and Osteryoung square wave voltammetry. Using a Britton–Robinson buffer at pH 6.0 linear range was from 4 to 50 μmol/L with a detection limit 0.024 μmol/L for differential pulse technique.

The primary goal of the present work was to develop and validate more precise, accurate, rugged and reliable method for the determination of moxifloxacin in human plasma which would be suitable for routine use in clinical practice with regards to the wide range of concentration in plasma samples and large number of samples needed to be analyzed in short time. We propose the procedure with minimal sample pre-treatment using direct injection into a chromatographic column and sensitive fluorescence detection in a micellar medium with enhanced sensitivity and accuracy of determination. For chromatographic separation a Hisep column was used. It can tolerate large number of small volume plasma injection retaining the small molecular weight analytes (drugs) which penetrate hydrophilic network, while large molecular weight substances (proteins) can not come into contact with hydrophobic phase and thus these molecules are not retained. According to manufacturer recommendations column can be used over a pH range 2–7 and content of organic modifier in mobile phase should be less than 15%. These restrictions can preclude elution of drugs which are highly hydrophobic. Moxifloxacin, due to its amphoteric character, in aqueous media is present in the ionic form in wide range of pH, so that separation on Hisep column is difficult. The problems involve broad tailing peaks, too early elution with low efficiency of separation and low solubility of the zwitterionic form in water. So, to achieve quality separation of moxifloxacin in a reasonable analysis time acceptable chromatographic factors must be adjusted. In order to keep capacity factors to be neither too low (bad resolution) nor to high (lack of sensitivity, long analysis time), the mobile phase composition and the pH must be optimized. Since both selectivity and retention vary upon varying the pH of the mobile phase, optimization usually involves simultaneous adjustment of both the composition and pH of the mobile phase. Thus, development of the direct injection method for the moxifloxacin determination in plasma requires mathematical optimization of the procedure. In this work we used fractional factorial technique of optimization.

2. Experimental

2.1. Reagents

Moxifloxacin hydrochloride standard (declared purity >99%), yellow powder, M_r = 437.9, was obtained from BayerPharma AG (Germany). Ofloxacin and pefloxacin standards were supplied by Hoechst (Frankfurt am Main, FRG) and Rhone Poulenc Rhorer (Paris, France), respectively. Acetonitrile,

chromatography purity grade, was obtained from Merck (Darmstadt, Germany). Sodium phosphate and phosphoric acid were p.a. purity grade products from Merck. Sodium dodecylsulfate ($M_r = 288.38$) p.a. purity grade was from Sigma (Germany). Water used for the preparation of mobile phase was obtained from EasyPure system (Barnstead, FRG). Human pool whole blood samples were obtained from the Department of transfusion of Clinical hospital "Dr. Dragisa Misovic", Belgrade.

2.2. Chromatographic system

Chromatographic analysis was performed on a modular HPLC system, Agilent 1100 Series (Waldbronn, Germany) consisted of binary pump, autosampler, column thermostat and fluorescence detector. Separation was achieved on a Supelco LC-Hisep column (USA), 150 × 4.6 mm, 5 μm. Mobile phase was prepared by mixing acetonitrile (A) and buffer solution consisting of 0.25 mol/L Na_3PO_4 with pH 3.0 adjusted with concentrated phosphoric acid (B), in a volume ratio 5:95. The mobile phase was delivered at 1.0 mL/min rate. Fluorescence detection was performed with excitation wavelength 290 nm and emission wavelength 500 nm.

2.3. Fluorescence measurements

The fluorescence intensity was measured on a Perkin-Elmer (Norwalk, USA) luminescence spectrometer equipped with a xenon lamp and a Dell model 110 L, computer working with WinLab software. All the measurements were performed in a 10 mm pathlength quartz cell thermostated at 25 ± 0.5 °C, with 5 nm band-widths both emission and excitation monochromators.

2.4. Standard solutions

Standard solution of moxifloxacin HCl was prepared by direct weighing of standard substance with subsequent dissolution in water. The concentration of the standard stock solution was 100.0 mg/L. Standard stock solution of ofloxacin was prepared by direct weighing of standard substance. The concentration of the stock solution was 50.0 mg/L. Working standard solution of moxifloxacin was prepared by diluting 3.0 mL of the stock solution to 100 mL in a volumetric flask and working standard solution of ofloxacin was made by diluting 1.0 mL of the stock solution to 100 mL in a volumetric flask, by doubly distilled water.

2.5. Plasma samples

Whole blood samples were centrifuged for 30 min at 2000 rpm. Supernatant was filtered through a Minisart plus syringe filter (0.2 μm pore size, Supelco) to remove remaining blood elements and high molecular weight proteins. Plasma samples, after spiking with appropriately diluted working solutions and displacing reagent were directly injected onto a Hisep column.

2.6. Optimization procedure

2.6.1. Mobile phase

During the optimization of mobile phase composition, aqueous 15% acetonitrile (ACN) was used first. Then the mobile phase containing 15% ACN and 0.5 or 1% triethylamine were prepared and the pH was adjusted with diluted H_3PO_4 to 6.0, 6.5 and 7.0. The mobile phase containing either HEPES or PIPES buffer (50 mmol/L) with a pH adjusted to 6.5 with diluted NaOH was also prepared. These mobile phases produced too low capacity factors. In the next optimization cycle the content of ACN was lowered down to 2.5% (v/v) and to this solution 97.5% (v/v) of either 0.25 mol/L Na_3PO_4, or Na_2HPO_4 or NaH_2PO_4 was added and pH was adjusted to 3.0 or 2.5 with conc. phosphoric acid. Further optimization was initiated with 7.5% and 5% (v/v) aqueous acetonitrile with the addition of 92.5% or 95% (v/v) Na_3PO_4 (pH 3.0 adjusted with conc. H_3PO_4), respectively.

2.6.2. Internal standard

Most so far reported methods for the moxifloxacin determination make use of internal standards. Enrofloxacin [4], lomefloxacin [7], ciprofloxacin [6], and ofloxacin [3] were employed in chromatographic determination of moxifloxacin in bio-fluids. In the optimization process two internal standards were tried: pefloxacin and ofloxacin in all stages of mobile phase composition optimization. Pefloxacin produced considerably lower resolution than ofloxacin so that the ofloxacin was the optimal internal standard.

2.6.3. Displacing reagent

Displacing reagent was used as a micellar phase to enhance the solubility of proteins and to minimize binding of moxifloxacin to plasma proteins. To aqueous 0.25 mol/L Na_3PO_4 various amounts of SDS were added to give the concentrations 5–15 mmol/L and pH was adjusted with conc. phosphoric acid to 3.0. Optimal displacing reagent consisted of the phosphate buffer and 10 mmol/L SDS.

2.6.4. Detection

Optimal response was obtained with excitation at 290 nm and emission at 500 nm. Intensity of the emission fluorescence curve varies with pH of solution. The fluorescence intensity of plasma samples is maximal between pH 3.0 and 5.0. The intensity decreases when increasing the pH of solutions (pH > 5). It is in accordance with Ocaña et al., findings [8] in the presence of SDS maximum micellar solubility of moxifloxacin is achieved at lower pH values (3–6). We chose the pH 3.0 as the optimal one for the determination.

2.6.5. Experimental design

On the basis of the optimization procedure the following factors were selected and tested in the experimental design: (A) volume percent of acetonitrile (2.5; 5.0; 7.5), (B) pH of the mobile phase (2.5; 3.0; 3.5), (C) concentration of Na_3PO_4 (0.1; 0.25; 0.3 mol/dm^3), (D) concentration of sodium dodecylsulfate (SDS) (5.0; 10.0; 15.0 mmol/dm^3). Each factor was examined

at three levels which were chosen smaller and higher than the nominal level as determined in preliminary optimization. Factor levels are given in parenthesis. The experimental design was a fractional factorial three-level design. Full factorial design would require $3^4 = 81$ experiments. In a fractional design we performed 27 experiments. Six responses were determined in each designed experiment: retention times, capacity factor, resolution, peak areas, number of theoretical plates and peak areas relative standard deviation, RSD (%). The following constraints were imposed on some responses: retention times should be less than 20 min, resolution higher than 1.2, peak areas RSD less than 5%. The analysis performed with the aid of the computer program Statistica v. 6 [13] reveals that the most important factors influencing resolution and retention time of chromatographic peaks are content of acetonitrile in mobile phase, pH of the mobile phase and less significant is the ionic strength (i.e. the concentration) of the phosphate buffer. The interaction between acetonitrile content and pH of the mobile phase is significant. This interaction was further examined by response surface method.

Experimental design also indicates that the best conditions for separation of moxifloxacin from internal standard (IS) and plasma components are at mobile phase composition: ACN: 0.25 mol/L Na_3PO_4 = 5:95, v/v%; pH 3.0 and SDS concentration, 10 mmol/L. Typical chromatograms of water matrix and plasma matrix samples spiked with moxifloxacin at 2.0 μg/mL, at the best conditions are shown in Fig. 1a and b.

The characteristics of the obtained chromatograms are given in Table 1.

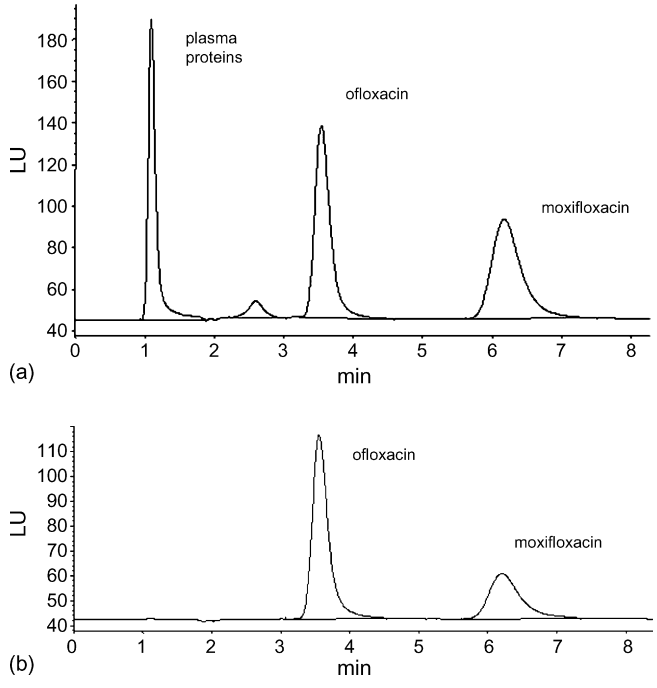

Fig. 1. Chromatograms of (a) plasma matrix sample and (b) aqueous matrix sample of moxifloxacin (2 μg/mL) and IS (ofloxacin) (1 μg/mL) at pH 3.0. [SDS] = 10 mmol/L.

Table 1
Chromatographic separation characteristics of moxifloxacin (2 μg/mL) and ofloxacin (1 μg/mL) analyzed under best conditions (ACN: 0.25 mol/L Na_3PO_4 = 5:95, v/v%; pH 3.0, [SDS] = 10.0 mmol/L)

Characteristics	Aqueous matrix sample		Plasma matrix sample	
	MFLX	OFLX	MFLX	OFLX
No of theoretical plates	1036	1292	1095	1340
Capacity	5.20	2.56	5.17	2.55
Resolution	4.53	-	4.63	2.39
Asymmetry	0.70	0.69	0.70	0.71
Retention time	6.203	3.556	6.174	3.546

2.7. Blank calibration curves

From standard stock solution the standard working solution containing 10.0 μg/mL of moxifloxacin was prepared by diluting with doubly distilled water. The working solution for calibration curve construction was prepared by diluting the aliquot of standard working solution with water to yield the concentration of 3.0 μg/mL. Aliquots of this solution were mixed with appropriate volume of displacing reagent and to each solution used for calibration graph, standard working solution of ofloxacin was added so that the final concentration of ofloxacin was 0.025 μg/mL and that of SDS 10.0 μmol/mL. The concentration range of moxifloxacin was 0.003–1.30 μg/mL (12 solutions).

2.8. Calibration graph for plasma

To 0.40 mL of plasma, standard working solution of moxifloxacin was added in a volumetric flask of 5 mL and the solution was filled with water to the mark. The concentration of moxifloxacin was 3.0 μg/mL. This solution was further subjected to the same procedure as for the blank calibration curves. The calibration graph was obtained by plotting the moxifloxacin to ofloxacin peak area ratio against the concentration ratio of moxifloxacin to internal standard (IS), i.e. ofloxacin.

2.9. Linearity

To test the hypothesis that the data used in linear model are homoskedastic (set of estimated variances of responses is homogeneous) Cohran's test was applied [14]. The test is based on the distribution of the random variable $G_{exp} = s^2_{max}(Y)/\Sigma s^2(Y_i)$, where $s^2_{max}(Y)$ is the highest obtained variance of the Y response (Y = peak area ratio). The variable G is a function only of the number of replications, n and total total number of measurements, k so that if the relationship $G_{exp} < G\alpha(n\text{-}1, k)$ is valid the hypothesis is true. At significance level $\alpha = 0.05$, $n = 3$ and $k = 12$, $G_{0.05}(2, 12) = 0.392$ and since experimentally obtained value $G_{exp} = 0.216$ the tested hypothesis is accepted.

2.10. Precision

The precision was expressed as the coefficient of variance CV (%) of six identically prepared and measured calibration

samples at each concentration level, during 1-day measurements series.

2.11. Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to ICH [15]: LOD = 3.3 × (s_b/a) where s_b is the standard deviation of the y-intercept of the calibration line and a is the slope of the calibration line. The limit of quantitation was calculated through the equation: LOQ = 10 × (s_b/a). To asses the lower limit of quantitation, five samples with decreasing concentration of moxifloxacin were injected six times. The calculated relative standard deviation of analyte to IS peak ratio was plotted against moxifloxacin concentration. We required the precision at the minimum quantifiable concentration less than 10%.

2.12. Recovery

Recovery was calculated through the equation:

$$\text{Recovery}(\%) = 100 \times \left(\frac{\text{slope of the plasma calibration curve}}{\text{slope of the water calibration curve}} \right).$$

2.13. Sample preparation

Tablets of moxifloxacin (nominally 400 mg, "Avelox", Bayer AG, Leverkusen, FRG) were used for oral administration to two healthy volunteers with no clinical signs of either acute or chronic illness. Conventional laboratory tests performed before individual dosage including standard hematology, creatinine clearance, plasma urea and bilirubin produced normal results. The trial was started after an overnight fast which continued for another 4 h after dosage. Blood samples were taken by cubital vein puncture 2 h after administration of a single dose of 400 mg tablet, and 2 h after repeated dose given next day. Blood specimens were allowed to clot at room temperature for 30 min and subsequently centrifuged at 2000 rpm for 10 min. The supernatants were stored at −20 °C until analysis. After reconstitution, the samples were treated in the same procedure as for the plasma calibration line. Owing to high dilutions of samples no clean up procedure for the column was necessary after injections.

2.14. Quality control samples and accuracy of the method

The quality control samples with low (1.5 μg/mL) middle (3.0 μg/mL) and high (5.0 μg/mL) concentration of moxifloxacin were prepared by spiking blank human plasma with appropriately diluted standard solution of moxifloxacin (100.0 μg/mL). This range of concentrations was based on moxifloxacin concentration found in previous pharmacokinetic studies when the drug was administered at 400 mg once a day [2]. QC samples were processed in the same way as those for plasma calibration curve. For precision and accuracy assay six replicates of each low, middle, and high quality control samples were analyzed. The mean standard deviation, RSD and accuracy were calculated. The accuracy was determined as a relative error bias calculated from the equation:

$$\text{Accuracy } (\%) = 100 \times \frac{(C_{\text{real}} - C_{\text{determined}})}{C_{\text{real}}}$$

where C_{real} denotes added amount of moxifloxacin while $C_{\text{determined}}$ is that calculated from the equation of calibration curve. Relative error of mean predicted concentration compared with nominal concentration was determined.

3. Results and discussion

Separation of ionizable analytes-acids and bases in terms of column efficiency, selectivity and retention depends on the pH of the mobile phase. Retention is usually improved on a non-polar column, by changing pH so that analytes are separated in their un-ionizable forms. Also, interaction between analyte and the silica surface of the column packing, that causes poor peak shape, could be minimized by choosing appropriate composition and pH of the mobile phase. To choose the optimal pH for moxifloxacin separation it is necessary to take into account that moxifloxacin is a weak heterocyclic amino acid with two protonation sites: one, corresponding to a proton gain on 3′-*N* secondary amino piperazinyl group and the other one, corresponding to proton loss from the carboxyl group. Thus, moxifloxacin may exist in solution in cationic, neutral, zwitter-ionic and anionic forms. Relative percentages of these forms in the solution depend upon the pH of the solution. Since the stationary phase best retains the neutral or zwitter-ionic form of moxifloxacin the pH interval for their maximum formation should be known. For this reason knowledge of iso-electric point of moxifloxacin is required, i.e. both dissociation constants in a particular solvent medium must be known. These constants were evaluated spectrophotometrically without and in the presence of SDS. Ten spectra of moxifloxacin in the pH interval 4.0–10.0 were taken in the wavelength range 250–350 nm. The spectra were digitized at every 5 nm. The experimental data: absorbance as the function of pH and wavelength, were subjected to non-linear regression analysis with the aid of the program Squad [16]. The results of calculation are given in Table 2.

Thus, isoelectric point of moxifloxacin is in neutral or weakly alkaline medium. In order to examine the best condition for the chromatographic separation of moxifloxacin from the soluble plasma constituents and internal standard, the chromatograms of moxifloxacin on Hisep column, in a mobile phase with

Table 2
Protonation constants of moxifloxacin anion ($MFLX^-$) defined according to equilibrium: K_1: $H^+ + MFLX^- \leftrightarrow HMFLX$ and K_2: $H^+ + HMFLX \leftrightarrow H_2MFLX^+$ determined spectrophotometrically at 298 K. $pI = \frac{1}{2}(pK_1 + pK_2)$.

Overall protonation constants	pK_1	pK_2	pI
Water	8.72 ± 0.09	6.16 ± 0.08	7.44
Water + SDS	8.80 ± 0.30	7.62 ± 0.3	8.21

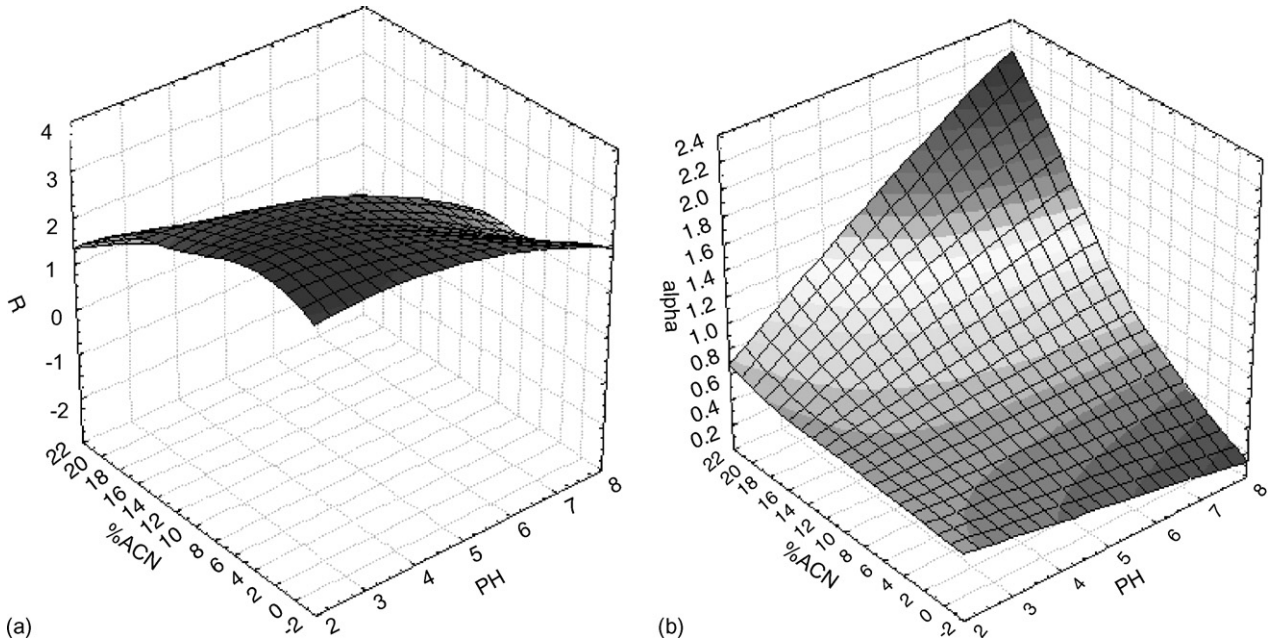

Fig. 2. (a) Response surface diagram of the dependence of relative resolution (R_{MFLX}/R_{OFLX}) on pH and volume % of ACN in the mobile phase. (b) Response surface diagram of the dependence of relative retention of moxifloxacin and ofloxacin on pH and volume % of ACN in the mobile phase.

varying percentage of acetonitrile and pH were recorded. The data involving the dependence of the relative resolution of moxifloxacin on volume percent of acetonitrile and pH are tabulated. These experimental data were subjected to the design of experiment analysis using the response surface technique. Statistical treatment of the uncoded experimental data was performed with the aid of the program Statistica v. 6.0. It has been found that the retention behavior of moxifloxacin can be approximated by a second-degree surface (Fig. 2a). Optimization of resolution shows that response surface possesses a relatively flat maximum situated between 10–15% ACN and pH between 3 and 4.5.

Optimization of relative retention (Fig. 2b) reveals the similar features as in the previous case. The response surface shows broad minimum located in the approximately the same area of factor space as in the previous case.

The optimal conditions as determined from the results of the experimental design were: pH of the aqueous phase in the mobile phase, 2.5–3.0, column temperature, 25 °C, acetonitrile to aqueous ratio in the mobile phase, 5:95 (v/v). Under the mentioned conditions the retention time periods were reasonable while the resolutions of moxifloxacin and ofloxacin (relative to un-retained component) were maximal. Also the best results of the peak area RSD were obtained.

The optimal pH for analysis was 3.0. Though the maximum fluorescence intensity is obtained at pH values around pH 4, other chromatographic responses (retention and resolution) were not acceptable in this pH interval, therefore, the pH of the aqueous phase in the mobile phase was decreased. Addition of SDS greatly improved the fluorescence intensity. Namely, the SDS creates a hydrophobic environment in which intramolecular quenching of fluorescence is hindered. Furthermore, non-polar part of SDS is bound to stationary phase with polar sulfonic group oriented toward mobile phase. Since both moxifloxacin and ofloxacin are in the cationic form (proton bound to amino moiety) they electrostaticaly interact with polar head of SDS thus improving retention. Decreasing the acetonitrile content in the mobile phase with other conditions being unchanged, increases the polarity of the mobile phase thus reducing the strength of the links with non-polar stationary phase and electrostatic interactions between analyte and polar head of SDS so that at some level of ACN concentration in the mobile phase the optimal retention is obtained.

The HPLC method under the optimal conditions was validated for linearity, selectivity, precision, accuracy, LOQ and LOD.

3.1. Validation

3.1.1. Linearity and regression analysis

Linearity in human plasma and mobile phase by HPLC with fluorescence detection was evaluated. The regression coefficients are acceptable ($R = 0.99987$ for mobile phase and $R = 0.99986$ for plasma). Results of regression analysis are given in Table 3. The calculated recovery was 92.5% which is acceptable for plasma samples.

3.1.2. Accuracy and precision

Accuracy determined during 1-day measurements of 12 concentration levels for six spiked plasma samples was expressed as relative error difference (RE) between measured and predicted (using regression equation) peak area ratio (Table 4). Precision was as relative standard deviation for each level for six repeated measurements evaluated. Furthermore, the analysis of independent low, middle and high quality control samples (QC) was used to determine intra-day precision and accuracy of the assay (Table 5).

Table 3
Regression analysis of the calibration lines

Parameter	Value	
	Aqueous matrix samples	Plasma matrix samples
Number of samples	12	12
Concentration range (μg/L)	1.0–1300.0	3.0–1300.0
Regression equation	$Y = aX + b$ (Y = peak area ratio, X = concentration of moxifloxacin to IS ratio)	
Slope	4.174 ± 0.004	3.860 ± 0.007
Intercept	$(1.54 \pm 1.0) \times 10^{-4}$	$(2.66 \pm 1.5) \times 10^{-3}$
Regression coefficient	0.99987	0.99986
Residual standard deviation	0.015	0.02
LOD (μg/L)	0.10	1.0
LOQ (μg/L)	1.0	3.0
Recovery (%)	92.5	

Table 4
Standard plasma curve validation of moxifloxacin at 12 concentration levels (six replications)

No	Mean peak area ratio ± SD	RSD (%)	RE (%)
1	0.185 ± 0.008	4.54	5.4
2	0.521 ± 0.018	1.56	10.9
3	0.802 ± 0.015	1.82	−3.7
4	1.303 ± 0.060	4.53	5.3
5	1.434 ± 0.006	0.39	0.75
6	1.720 ± 0.026	1.49	−0.75
7	1.903 ± 0.023	1.22	2.1
8	2.575 ± 0.098	3.81	−0.7
9	2.957 ± 0.037	1.26	−4.2
10	3.449 ± 0.083	2.41	1.7
11	3.841 ± 0.050	1.30	−0.26
12	4.881 ± 0.090	1.82	5.3

Table 5
The results of the analysis of the quality control samples

Nominal concentration (μg/mL)	Predicted concentration ± SD[a] (μg/mL)	RSD(%)	RE (relative error)
QC low, 1.5	1.48 ± 0.008	0.54	−1.33
QC middle, 3.0	3.08 ± 0.011	0.36	+2.7
QC high, 5.0	4.91 ± 0.040	0.82	−1.8

RE = 100 × (predicted − nominal conc.)/nominal conc.
[a] n = six replicate measurements.

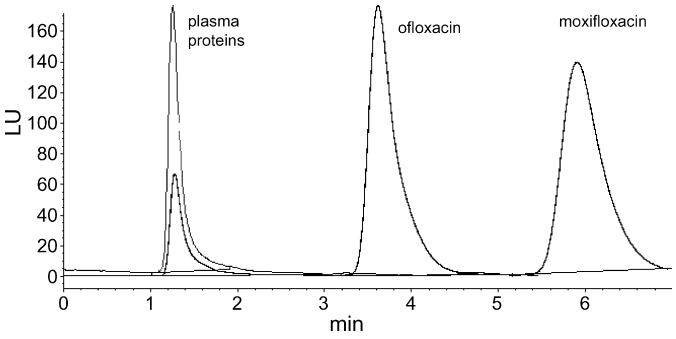

Fig. 3. Typical chromatograms of moxifloxacin and IS (ofloxacin) in plasma sample (dashed line) 2 h after administration of 400 mg Avelox tablet. Blank plasma: full line.

Overall mean precision (RSD) ranged from 0.4 to 4.5% while the accuracy ranged from −1 to 3%. The inter-day accuracy was evaluated by comparing the slopes of plasma calibration curves independently prepared on 10 consecutive days (Table 6). The obtained results indicated that the method was reliable, reproducible and accurate.

3.1.3. Selectivity

Blank drug free plasma was analyzed and no interfering peaks were observed at the retention times of the fluoroquinolones (Fig. 3). The selectivity was further assessed using blank patient samples collected immediately prior to administration of moxifloxacin. No interfering peaks were found at the retention times of either moxifloxacin or ofloxacin.

3.1.4. Stability tests

Stability tests of moxifloxacin in plasma was carried out at three concentration levels (low, middle and high) with six equally prepared samples et each level. The conditions examined were: three freeze–thaw cycles, storage of plasma samples at −20 °C for the period of 3 months. The conditions mentioned did not change the concentration levels of respective analyte significantly (e.g. more than of 5%).

3.1.5. Application

The method was applied to determination of mean plasma concentration of moxifloxacin after oral administration of 400 mg of Avelox tablets to two healthy volunteers. The results of analysis are given in Table 7.

These results are in accordance with literature data [2,5]. Due to the excellent separation efficiency the proposed method is suitable for complex mixture of fluoroquinolones determination. In Fig. 4 the chromatogram of the mixture of quinolones: pefloxacin, fleroxacin, ofloxacin, ciprofloxacin and moxifloxacin is resolved with the analytical method. Thus it

Table 6
Inter-day validation of moxifloxacin determination by measuring the slope of the calibration line

Day	Slope of the calibration curve					
	0	2	4	6	8	10
Aqueous matrix	4.174 ± 0.004	4.150 ± 0.002	4.000 ± 0.002	4.200 ± 0.004	4.000 ± 0.004	4.250 ± 0.004
Plasma matrix	3.860 ± 0.007	3.818 ± 0.008	3.685 ± 0.01	3.950 ± 0.01	3.803 ± 0.005	3.950 ± 0.008

Table 7
Results of analysis of patients plasma samples after administration of Avelox tablets. Concentrations are expressed in μg/mL ± SD

Sample	First day	Second day (repeated dose)
1	3.50 ± 0.10	4.95 ± 0.11
2	3.20 ± 0.15	5.11 ± 0.09

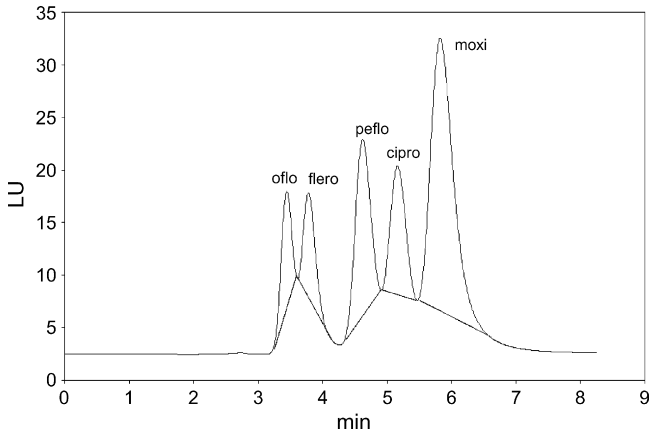

Fig. 4. Chromatogram of mixture of fluoroquinolones: ofloxacin (oflo), fleroxacin (flero), pefloxacin (peflo), ciprofloxacin, (cipro) and moxifloxacin (moxi) with mobile phase containing acetonitrile: 0.25 mol/L Na_3PO_4 = 5:95, v/v%; pH 3.0, [SDS] = 10 mmol/L.

would be possible to determine also metabolites of quinolones in plasma samples.

4. Conclusion

Analytical method for the determination of moxifloxacin in plasma samples with fluorescence detection was developed and validated. The proposed direct injection into shielded LC column enabled the determination of moxifloxacin with the following advantages: (a) simple and effective sample preparation, (b) analysis time less than 10 min/sample, (c) reproducible recoveries. Due to excellent separation efficiency the method would be possibly suitable for more complex mixtures of fluoroquinolones (and their metabolites or degradation products) determination, which has not been tested yet. The method is reliable, rugged and suitable for routine clinical analysis.

References

[1] J.A. Barman Balfour, L.R. Wiseman, Drugs 57 (1999) 363.
[2] H. Stass, A. Dalhoff, D. Kubitza, U. Schühly, Antimicrob. Agents Chemother. 42 (1998) 2060.
[3] S.H.Y. Wang, J. Phar. Biomed. Anal. 7 (1989) 1011.
[4] V. Samanidou, E.A. Christodoulou, I.N. Papadoyannis, Curr. Pharm. Anal. 1 (2005) 283.
[5] H. Stass, A. Dalhoff, J. Chromatogr. B 702 (1997) 163.
[6] A. Dalhoff, H. Stass, Drugs 58 (1999) 239.
[7] T. Lemoine, D. Breilh, D. Ducint, J. Dubrez, J. Jougon, J.F. Velly, M.C. Saux, J. Chromatogr. B 742 (2000) 247.
[8] J.A. Ocaña, F.J. Barragán, M. Callejón, Analyst 125 (2000) 2322.
[9] J.G. Möller, H. Stass, R. Heinig, G. Blaschke, J. Chromatogr. B 716 (1998) 325.
[10] K. Vishwanathan, M.G. Bartlett, J.T. Stewart, J. Pharm. Biomed. Anal. 30 (2002) 961.
[11] B.B. Ba, R. Etienne, D. Ducint, C. Quentin, M.C. Saux, J. Chromatogr. B 754 (2001) 107.
[12] N. Erk, Anal. Bioanal. Chem. 378 (2004) 1351.
[13] Statistica v. 6. StatSoft. Tulsa (USA), 2001.
[14] Statistica Electronic Manual. Statsoft Inc. Tulsa (USA). 2001.
[15] "Validation of analytical procedures: Methodology", ICH Gudeline Q2B. Federal Register 62, No. 96 (1997). Pp 27463–27467;
M. Thompson, S.L.R. Ellison, R. Wood, Pure Appl. Chem. 74 (5) (2002) 835.
[16] D.J. Leggett (Ed.), Computational Methods for the Determination of Formation Constants, Plenum Press, New York, 1985, p. 159.