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Optimization of separation and determination of moxifloxacin and its related substances by RP-HPLC☆

Predrag Djurdjevic[a,*], Andrija Ciric[a], Aleksandra Djurdjevic[b], Milena Jelikic Stankov[c]

[a] Chemistry Department, Faculty of Science, 34000 Kragujevac, P.O. Box 60, Serbia
[b] Medicines and Medical Devices Agency of Serbia, 11000 Belgrade, Serbia
[c] Analytical Chemistry Department, Faculty of Pharmacy, 11000 Belgrade, Serbia

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ABSTRACT

A RP-HPLC method for the separation and determination of impurities of moxifloxacin, in its pharmaceutical forms as well as moxifloxacin degradation products, was developed with the aid of DryLab® software and chemometric (response surface) approach. The separation of four synthesis-related impurities was achieved on a Waters C_{18} XTerra column using a mobile phase of (water + triethylamine (2%, v/v)): acetonitrile = 90:10 (v/v%); the pH of water phase being adjusted with phosphoric acid to 6.0. Flow rate of the mobile phase was 1.5 ml/min and UV detection at 290 nm was employed. The column was thermostated at 45 °C. The resolution between the two least resolved impurity peaks was in average, $R_{s,min} > 1.5$. Method validation parameters indicate linear dynamic range 0.2–2.0 μg/ml with LOQ ca. 0.20 μg/ml and LOD ca. 0.05 μg/ml for all analytes.

The method was applied for the impurities determination in drug tablets and infusion (Avelox®, Bayer AG) and for degradation products determination in a stability study of moxifloxacin. The impurity content in the tablets and infusion was quantified as 0.1% of total drug. Two degradation products were noted under hydrolytic conditions. The method can also be used for rapid and accurate quantification of moxifloxacin hydrochloride in its tablets during stability testing.

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-quinolone carboxylic acid hydrochloride] (MOX) is a synthetic antibacterial agent active against Gram-negative and some Gram-positive bacteria. Its pharmaceutical formulations involve tablets and infusions (Avelox®, Avalox®, Tovan®, Bayer AG). It is synthesized by various procedures which commonly involve two main phases: (a) synthesis of quinolone nucleus and (b) introduction of various substituents [1,2]. Bayer AG (Leverkusen, Germany) has patented several synthetic processes [3], the main one being outlined in Scheme 1 [3,4].

During the synthesis developed by Bayer AG, not only un-reacted difluoro compound but also its related analogues: (i) 1-cyclopropyl-7-[(*S*,*S*)-2,8-diazabicyclo[4.3.0]non-8-yl]-6,8-difluoro-1,4-dihydro-4-oxo-3-quinolone carboxylic acid [6,8DF], (ii) 1-cyclopropyl-7-[(*S*,*S*)-2,8-diazabicyclo[4.3.0] non-8-yl]-6,8-dimethoxy-1,4-dihydro-4-oxo-3-quinolone carboxylic acid [6,8DM], (iii) 1-cyclopropyl-7-[(*S*,*S*)-2,8-diazabicyclo[4.3.0]non-8-yl]-8-fluoro-6-methoxy-1,4-dihydro-4-oxo-3-quinolone carboxylic acid [6M8F], and (iv) 1-cyclopropyl-7-[(*S*,*S*)-2,8-diazabicyclo[4.3.0]non-8-yl]-8-ethoxy-6-fluoro-1,4-dihydro-4-oxo-3-quinolone carboxylic acid [6F8E] (Scheme 2) are usually carried over in small quantities into bulk MOX [4,5]. Hence, the drug may contain impurities or to degrade during formulation process and stability testing under accelerated and long term storage conditions. Identification limits must be established for each impurity in accordance to ICH guidelines [6] and if the limit level is exceeded the impurity must be identified and quantified [7,8].

Several HPLC methods have been reported for moxifloxacin determination in its pharmaceutical forms and biological matrices [9–11]; no one was stability indicating. Isolation and identification of synthesis-related impurities was dealt with in some papers. Kumar et al. [12], isolated and structurally characterized four impurities in bulk moxifloxacin: 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[(*S*,*S*)-*N*-methyl-2,8-diazabicyclo(4,3,0)non-8yl]-4-oxo-3-quinoline carboxylic acid, methyl-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[(*S*,*S*)-2,8-diazabicyclo(4,3,0)non-8-yl]-4-oxo-3-quinoline carboxylate, 1-cyclopropyl-6-fluoro-1,4-dihydro-8-hydroxy-7-[(*S*,*S*)-2,8-diazobicyclo(4,3,0)non-8-yl]-4-oxo-3-quinoline carboxylic acid, and 1-cyclopropyl-6,7-difluoro-

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* Corresponding author. Fax: +381 34 335 040.
E-mail address: preki@kg.ac.yu (P. Djurdjevic).

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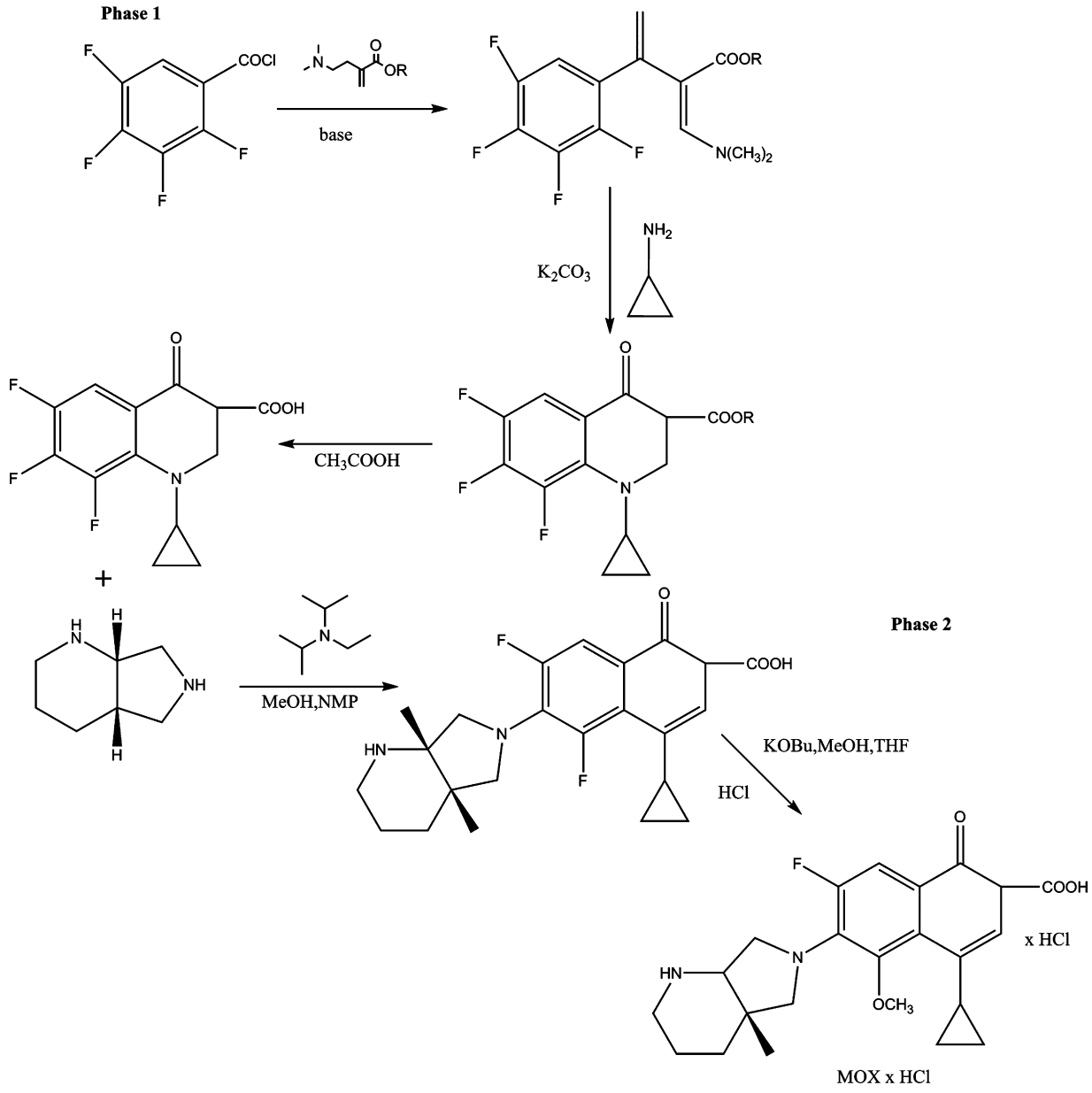
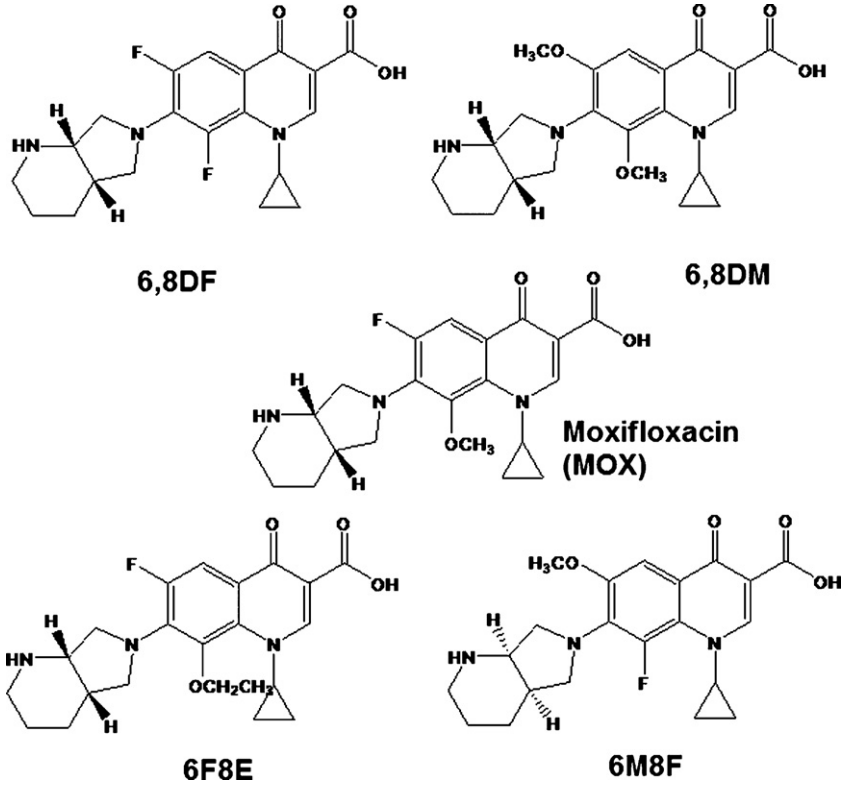


Scheme 1. Synthetic route of moxifloxacin HCl [3].

8-hydroxy-4-oxo-1,4-dihydro-3-quinoline carboxylic acid. The different structures of impurities found by Kumar et al. and those stated by Bayer AG could be attributed to different sythetic routes [2,3]. Motwani et al. [13], found three synthesis-related impurities by HPTLC method. The drug was also subjected to acid and alkali hydrolysis, oxidation, dry and wet heat treatment and photo degradation. Significant degradation was observed under hydrolytic conditions (seven products) while under other conditions degradation was milder (three products). Salem et al. [14] applied two stability-indicating methods, densitometric TLC and derivative spectrophotometry for the determination of lomefloxacin, moxifloxacin, and sparfloxacin in the presence of their acid degradates. The main degradant, a decarboxylated product was separated by TLC. Wei et al. [15] characterized some moxifloxacin impurities by spectrophotometric method. To our knowledge, no method for separation and determination of synthetic impurities of moxifloxacin based on chemometric approach was described in literature.

The main goal of the present investigation was to obtain optimal separation of components in a reasonable analysis time by adjusting acceptable chromatographic factors. The methods achieving this goal are based on the optimization of the mobile phase composition, pH, additive concentration, flow rate, type of chromatographic column, temperature and buffer selection. Simultaneous optimization of that many parameters requires computer oriented chemometric approach in order to simplify and accelerate the optimization process. In the present study a computer simulation software DryLab® was used in developing and optimizing a reverse-phase HPLC separation of moxifloxacin and its related impurities and degradation products of moxifloxacin [16–18]. Scouting analytical runs are required for DryLab® calculations since they train ("calibrate") the software. With the input data of training runs the software evaluates the resolution, R_s as a function of one or two chromatographic parameters for each peak pair. A "critical resolution map" is produced by plotting smallest value of resolution of any two critical peaks as a function of one or two varied experimental parame-



Scheme 2. Structure of moxifloxacin and related impurities with abbreviations.

ters revealing not only the optimum chromatographic conditions but also the robust regions of an HPLC method [16]. The predictive power of DryLab® software is based on Eq. (1) derived from the thermodynamic considerations of chromatographic process [19]:

$$\ln k = A + B \cdot D + C \cdot \Delta A + D(K^e - 1)V^{2/3}\gamma + E + \ln(RT/P_0V) \qquad (1)$$

This equation involves six energetic contributions—electrostatic forces, van der Waals forces, cavity term, correction term for non-planar surfaces, dielectric constant term and entropy of condensation term. The meaning of individual terms in Eq. (1) is:

k: capacity factor
A: experimental accessible constant
$\Delta A = (A_S + A_L - A_{SL})$ solvophobic contact surface area in solute (S)–ligand (L) complex
BD: electrostatic term (D approx. = 1)
V: molar volume of the solvent
K^e: molecular parameter of the solute
P_0: atmospheric pressure
γ: surface tension
T: temperature
R: gas constant
$C\,\Delta A$ (=$N\gamma\,\Delta A/RT$): cavity reduction term
N: Avogadro's number
E: van der Waals term

Eq. (1) describes the influence of the composition of eluent on retention based on the surface tension, γ, which is proportional to (100 − %B = %A), the amount of water in the mobile phase, the temperature T, molecular properties of sample and chemically bonded ligand, the contact surface area between ligand and solute ΔA and electrostatic properties such as buffer concentration. The DryLab® software allows for simultaneous optimization of one or two variables with input parameters, retention time, peak area and peak-width under chosen chromatographic conditions (column, temperature, gradient or isocratic elution) and is visualizing peak movements, which are essential to understand robustness of an HPLC method. The resolution map of one-dimensional optimization (1D) is two-dimensional graph whereas the resolution map of a two-dimensional optimization (2D) is a three-dimensional contour plot in which the critical resolution as a third dimension is color coded. Sliding a cross-hair marker to the region of maximum critical resolution one is able to optimize values of parameters and simulated chromatogram can be obtained, so that best experimental conditions can be easily identified.

To study the individual contribution of chromatographic factors to resolution of the peaks the response surface method was used [20]. The overall experimental response, R_x, is taken as a function of independent variables, %B, pH, T and %TEA (factors). The response, overall resolution, R_x is defined as a sum of individual resolutions between pairs of critical peaks [21]. Hence, the R_x will increase as analytical performance improves. Plot of R_x on factor space is the response surface. The region close to the extremum, a "nearly stationary region" can be suitably described by using second order polynomial:

$$R_x = b_0 + \sum_{i=1}^{f} b_i x_i + \sum_{i=1, j>1}^{f} b_{i,j} x_i x_j + \sum_{i=1}^{f} b_{ii} x_i^2 + \varepsilon \qquad (2)$$

where f is the dimension of the factor space and ε is the error associated to the R_x, which is assumed to be normally distributed. We determined the factors corresponding to optimum response (nearly stationary space) employing the software StatSoft Statistica v.6 [22].

2. Materials and methods

2.1. Reagents

All reagents were HPLC reagent grade purity unless stated otherwise. Moxifloxacin hydrochloride and its synthetic impurities and

ofloxacin as internal standard (IS) were used as certified reference compounds (Bayer AG, Germany) for quantitative analysis. Avelox® tablets (400 mg) and infusion (400 mg/250 ml) were products of Bayer Health Care, Leverkusen, Germany.

Acetonitrile was obtained from J.T. Baker (Deventer, The Netherlands) while ortho-phosphoric acid, sodium hydroxide and hydrochloric acid were products of Merck (Darmstadt, Germany). Triethylamine (99.5%, v/v) was obtained from Fluka (Buchs, Switzerland). HPLC grade water was produced by using Milli-Q water purification system (Millipore, Milford, USA) and was used for preparation of all solutions and reagents.

2.2. Apparatus

A HPLC system (Shimadzu, Kyoto, Japan) consisted of degasser DGU-20A$_3$, analytical pumps LC-20AT, 7125 injector and SPD-M20A diode array detector and CBM-20A system controller as well as Agilent 1100 series HPLC system (Agilent, CA, USA) comprising quaternary pump, injector, degasser and diode array UV detector were used for analysis. A reversed-phase Supelco (Bellefonte, PA, United States) ABZ C18 (50 mm × 4.6 mm, particle size 5 μm) and XTerra C18 (Waters, Milford, USA) column (50 mm × 4.6 mm, particle size 5 μm) were used for separation. The chromatographic data were processed using LC Solution computer software (Shimadzu) and ChemStation software (Agilent).

For the disintegration of the Avelox tablets and acceleration of dissolution, an ultrasonic bath (Bandelin Sonorex Super, Model RK 512H) was used.

2.3. Chromatographic conditions

The chromatographic separations were performed using either Supelco ABZ or Waters XTerra column using a mobile phase, water (+2% triethylamine): acetonitrile 90:10 (v/v%), the pH of the aqueous phase being adjusted to 6.0 with phosphoric acid, with the flow rate of mobile phase of 1.5 ml/min at 45 °C. The samples were monitored at 290 nm. 20 μl volume of sample was injected into HPLC system.

2.4. Analytical procedure

2.4.1. Stock solutions

For the preparation of stock solutions of moxifloxacin, ofloxacin and five impurities 0.1% phosphoric acid was used as solvent and diluent. The concentration of moxifloxacin was 10.01 mg/ml, ofloxacin was 0.206 mg/ml, 6M8F was 55.0 μg/ml, 6F8E was 51.0 μg/ml, 6,8DM was 51.0 μg/ml, 6,8DF was 52.0 μg/ml.

2.4.2. Standard solutions

Working standard solutions were prepared by dilution of appropriate volumes of stock solutions to 10 ml. For DryLab® simulations the mix solution contained moxifloxacin and its related impurities at the same concentration level, 10 μg/ml. The working solutions for optimization study contained 200 μg/ml of moxifloxacin, 1.0 μg/ml of ofloxacin as internal standard (IS) and 1.0 μg/ml of all impurities.

2.4.3. Sample preparation

Three tablets were accurately weighed (to obtain the average mass of one tablet) then finely powdered and 273.2 mg of homogenized powder was transferred to 25 ml volumetric flask. Approximately 20 ml of the diluent was added and the mixture was sonicated for 15 min. The mixture was then diluted to volume with the diluent. The solution was filtered through a 0.22 mm nylon filter. Exactly 175 μl of filtrate, equivalent to 200 μg/ml of moxifloxacin, was pipetted using an Eppendorf pipette into a 5 ml volumetric flask. Solutions in two flasks were diluted to the mark with diluent and one flask was used for analysis of the impurities while the other was used for degradation studies. Degradation was carried out under hydrolytic conditions in acid (0.1 M HCl), neutral (water medium) and base (0.1 M NaOH) medium. Solutions were left at 50 °C for 3 h. Oxidation stress was performed in 3% H_2O_2 solution; the solution was left for 6 h at room temperature, while photolytic stress was done in light chamber equipped with lamp bank with two Osram UV lamps. Total 840 Wh/m^2 of irradiation for 3 h, was used.

0.625 ml of infusion was transferred to a 5 ml volumetric flask and diluted with the diluent to mark. This solution was used for impurity determination.

The degradation of bulk moxifloxacin was carried out in parallel, in the same way as described above.

Before analysis to all solutions the same quantity of internal standard (ofloxacin) was added so that its final concentration was 1 μg/ml.

2.5. Method development

The method development started with ABZ column and buffered (pH 6.0 adjusted with H_3PO_4 + 2%TEA in water phase) water (A)-tetrahydrofuran, THF (B) and water (A)–methanol, MeOH (B) mixture as the mobile phases with linear gradient from 0 to 50% B in 20 and 40 min. No separation of peaks was noted with THF, while with MeOH no better resolution than R_s = 1.1 between critical pairs of peaks could be achieved. In further set of experiments the mobile phases were exchanged to water (A)–acetonitrile, ACN (B) mixture. All experimental data: retention times, peak areas and widths, temperature, column information and instrumental data, were entered into DryLab® software. To obtain 2D resolution map three combinations of parameters were considered: tG vs. eluent composition (ACN:H_2O), tG (gradient time) vs. column temperature; and eluent composition vs. pH. Other possible combinations were not considered. Optimization was started with gradient runs. Then, retention data from gradient runs were used to predict isocratic separation. Before starting analytical runs the dwell volume of HPLC systems was determined. The dwell volume must be determined to be able to make method transfer from one to another HPLC system. First, HPLC column was removed and injector and detector were short connected by piece of tube. Gradient run was made with acetonitrile as solvent A and 0.1% acetone solution in acetonitrile as solvent B. Linear 10 min gradient with 0–100%*B* was used with flow rate of 2 ml/min. A plot of %*B* vs. tG was made and from the retention time at midpoint of the gradient the dwell volume was calculated [18]. For Agilent system the dwell volume was 1.7 ml and for Shimadzu system, 2.7 ml. These data were entered into a program to relate sample retention to gradient composition at the column. The optimization of analytes separation was started with two linear gradient runs: tG = 30 min and tG = 60 min; %*B* run from 5 to 50% in the DryLab® mode LC-Gradient. Temperature was 30 °C and pH of water phase was 5.5. Along with retention times and peak areas the baseline peak-widths were also entered into the program. The resulting 1D resolution map is shown in Fig. 1.

Identification of peaks (peak tracking) was ensured by co-injecting the standards. From Fig. 1 the optimal %*B* for isocratic work can be easily identified to be in the range 10–13% ACN. Further, the column temperature was optimized by four linear gradient runs with tG = 30 and 60 min, %*B* from 5 to 20% and temperatures 30 and 60 °C (DryLab® mode LC-RP Gradient/Temperature). From 2D resolution map presented in Fig. 2 the optimal temperature range was identified at 45–47 °C.

Bearing in mind that all analytes are ionizable amphoteric substances one may expect the strong influence of pH on separation. Experimental design for pH optimization is presented in Scheme 3.

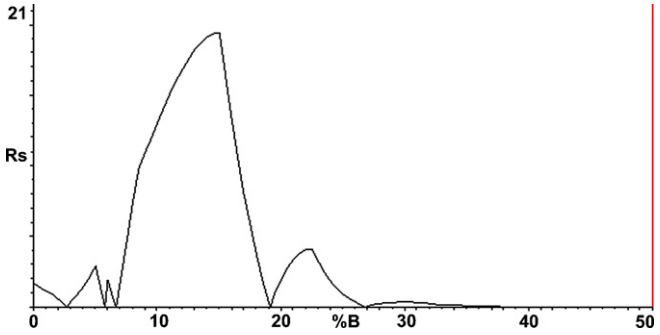


Fig. 1. 1D resolution map obtained in tG/*%B* optimization at pH 5.5 and $T = 30\,^{\circ}C$.

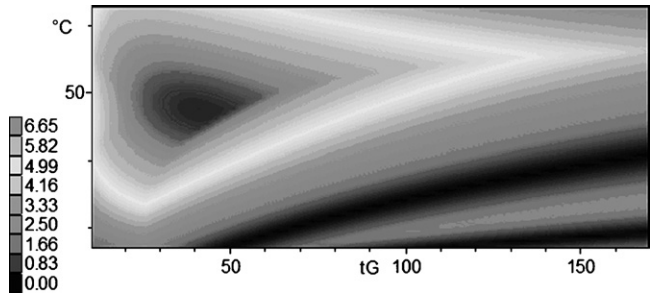
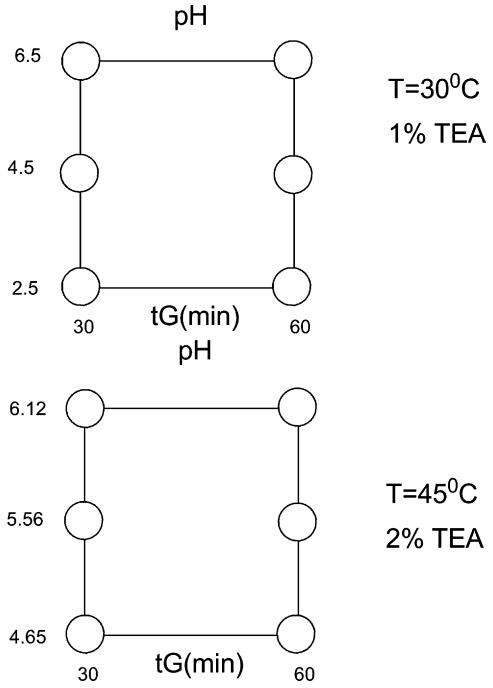


Fig. 2. 2D resolution map in tG/temperature optimization at pH 6.12.



Scheme 3. Experimental design for LC-tG/pH optimization.

To find optimal pH first, six screening gradient runs were carried out with tG = 30 and 60 min, *%B* varying from 5 to 20% and pH values at 2.5, 4.5 and 6.5 (DryLab® mode LC-RP Gradient/pH). The training (scouting) set of screening chromatograms is presented in Fig. 3. These runs indicated that the optimal area of pH is above 4.0.

Table 1
A comparison of predicted and experimental retention data for the optimized separation of model compounds (11% B; pH 6.10).

Peak I.D.	Retention time (min)		Resolution	
	DryLab	Experimental	DryLab	Experimental
6,8DF	3.16	3.20	5.55	5.50
MOX	4.65	5.00	4.09	4.00
6,8DM	6.16	6.20	2.36	2.41
6M8F	7.24	7.14	3.66	3.00
6F8E	9.34	9.08	n/a	n/a
Average standard error	0.10		0.20	

Though satisfactory values of R_s can be obtained at lower pH values (2.5), analysis time is too long and conditions are not robust. Thus, additional six gradient runs were made at pH 4.65, 5.56 and 6.12 under the same gradient conditions.

In this way the recommendation [18] not to change the pH for more than one unit near pK_a was followed. The dissociation constants of moxifloxacin are $pK_{a,1} = 6.25$ and $pK_{a,2} = 9.29$ [23]. Thus, the chosen pH interval adequately brackets the area near the first dissociation of moxifloxacin. The temperature was set at 45 °C and flow rate at 1.5 ml/min.

Inspecting the chromatograms one may see that at lower pH values the separation between 6,8DM and 6M8F is adequate but resolution between MOX and 6,8DF is unacceptable. Increasing the pH generally improves the resolution and MOX and 6,8DF adequately separate, but fine tuning between *%B* and pH is needed to improve resolution between critical pair 6,8DM and 6M8F.

From 2D robust resolution map it can be seen that optimal pH is in the range 5.8–6.1. From this map it can also be seen that Δt_R is much lower than 25% of tG so that isocratic mode might be possible. Changing DryLab® conditions from 2D resolution map to isocratic mode, from 2D resolution map optimal conditions are identified at pH 6.0 and *%B* = 9.8%. The isocratic resolution map and calculated (simulated) chromatogram are shown in Fig. 4.

A simulated isocratic run is compared with experimentally obtained isocratic chromatogram under optimal conditions found in DryLab® simulations, and results are presented in Table 1.

Data in Table 1 show that DryLab® accurately predicts retention of analytes. The calculated number of plates for moxifloxacin was 9654.

From DryLab® simulations it is evident that *%B*, pH and temperature show strong influence on retention and separation of the analytes. Nearly optimum condition required pH around 6, *%B* about 10%, and temperature higher than 40 °C. Though the resolutions of critical pairs under these conditions are satisfactory, significant tailing was obtained for moxifloxacin and 6-fluoro-8-ethoxy analytes. Therefore we exchanged ABZ column for XTerra which provides more symmetrical peaks. There was no significant difference in optimal chromatographic parameters between these two columns. DryLab® simulation is based on thermodynamic consideration of chromatographic process and it is desirable to see how phenomenological, i.e. statistical approach compares with thermodynamical. Further optimization was performed on XTerra column using factor analysis and response surface methodology with initial values of factors taken from DryLab® analysis. Response surface methodology would enable statistical modeling and quantification of factor effects on the chromatographic process. The following factors were examined: pH (X_1), *%B* (X_2), temperature, %TEA (X_3). Levels of these factors together with their coded values for customized central composite design are summarized in Table 2.

Coding of factors was performed according to formulae:

$$X_{coded} = \frac{(X_{real} - X_{average})}{(range/2)}$$

Table 2
Chromatographic factors and their coded values.

pH (X_1)	4	4.5	5	5.5	6	6.5	7	7.5	8
Coded values	−2	−1.5	−1	−0.5	0	0.5	1	1.5	2
%ACN (X_2)			8	9	10	11	12		
Coded values			−2	−1	0	1	2		
%TEA (X_3)				1	2	3			
Coded values				−1	0	1			

$$X_{average} = \frac{(X_{max} + X_{min})}{2}$$

$$range = X_{max} - X_{min}$$

In each experiment overall resolution, i.e. the sum of all resolution of individual resolutions between pairs of peaks was used as response. Between two peak resolution $R_{s1,2}$ was calculated using the formula [24]:

$$R_{s1,2} = \frac{1}{4}\left(\frac{k'_2}{k'_1} - 1\right)\sqrt{N_p}\left(\frac{k'_1}{k'_1 + 1}\right) \quad (3)$$

where N_p is number of theoretical plates for the drug, k_1 is a capacity factor for peak 1 and k_2 for peak 2. The experiment was designed as customized central composite face centered design with replications. In order to reduce the number of experiments the temperature was set to 45 °C, as optimal value determined from DryLab® simulation. The complete coded design is given in Table 3. The factors are designated as X_1 = pH, X_2 = %ACN, X_3 = %TEA.

The design consists of 37 runs with some fractional factorial and star points with three replicates at central point and some other replicate points, so that pure error can be estimated. The calculations were performed by using a program Statistica v. 6.

Initial ANOVA calculation indicated 11 coefficients in a quadratic model of which four are linear, three are quadratic and three are interactions. Since, calculated *p*-statistics was higher than 0.05 for X_1X_3 interaction coefficient, in a second calculation cycle this coefficient was omitted and included in the lack-of-fit. ANOVA analysis (Table 4) gave mean lack of fit of 4.4 which suggest applicability of quadratic model.

In Table 5 coefficients from Eq. (2) are presented together with standard error.

The calculated *p*-statistics indicate that eight coefficients are important, the most important being quadratic pH term. Also, pH and %ACN interaction is important in the model, thus, changes in both pH and organic modifier of mobile phase simultaneously exert effect on resolution. Careful regulation of both is needed in order to achieve good separation. Quantity of TEA and its interaction with ACN is less important.

Calculated response surface is shown in Fig. 5. It has relatively broad maximum indicating favorable robustness of the method.

The optimal values were searched using non-linear least square method. The optimal calculated values were: pH 6.0, %ACN = 10%,

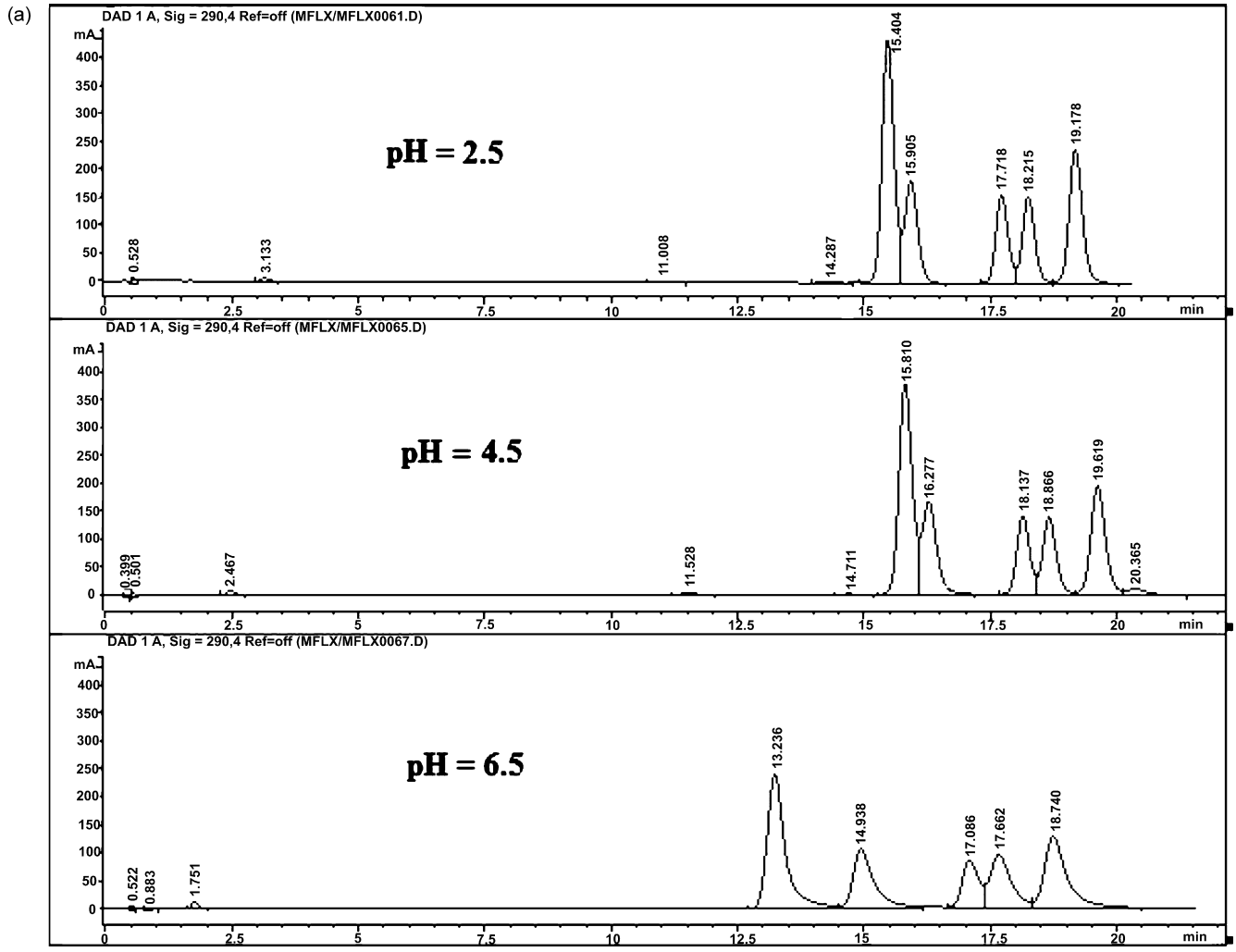


Fig. 3. Training set of chromatograms for screening LC-gradient tG/pH optimization. T = 30 °C. (a) tG = 30 min; (b) tG = 60 min.

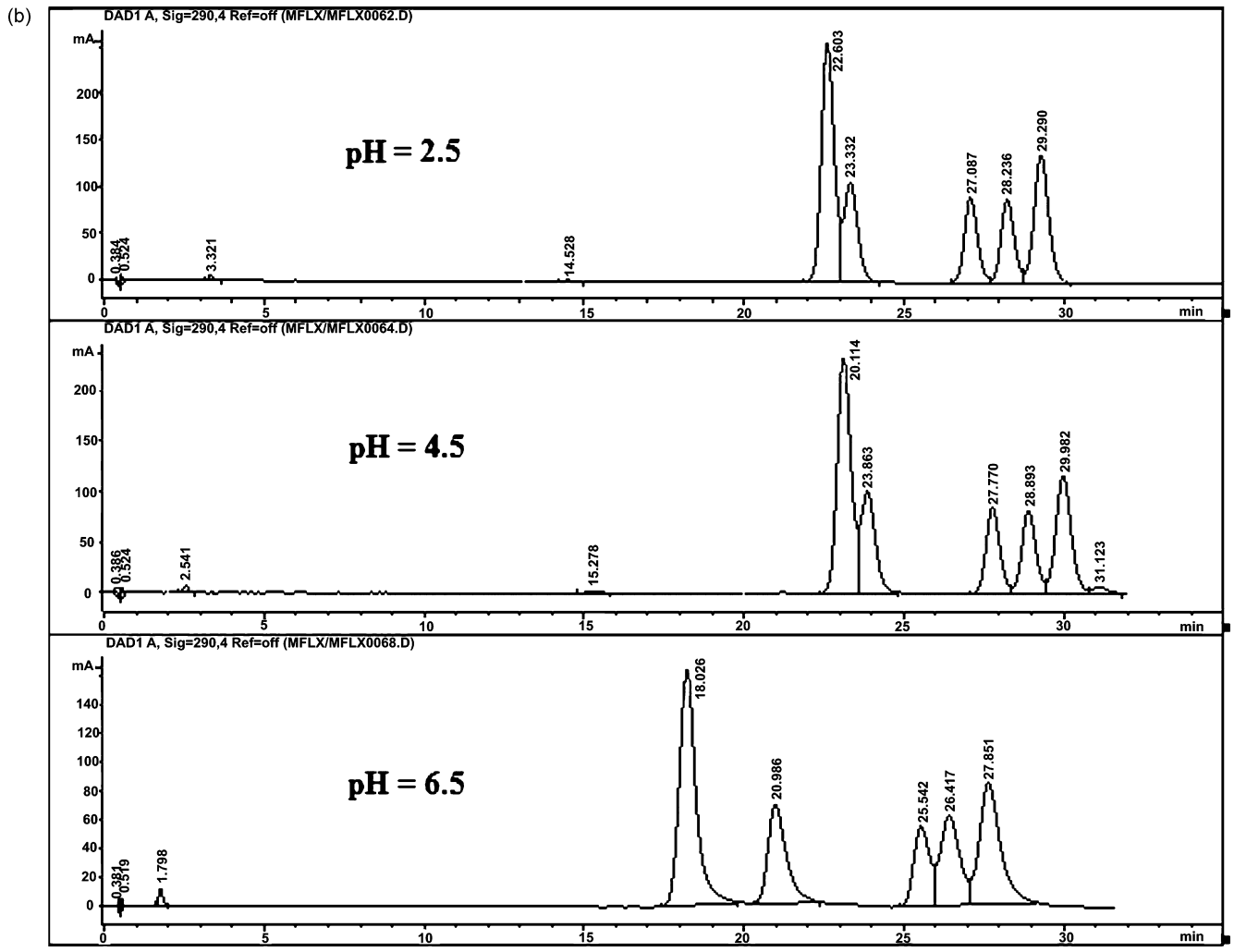


Fig. 3. (*Continued.*)

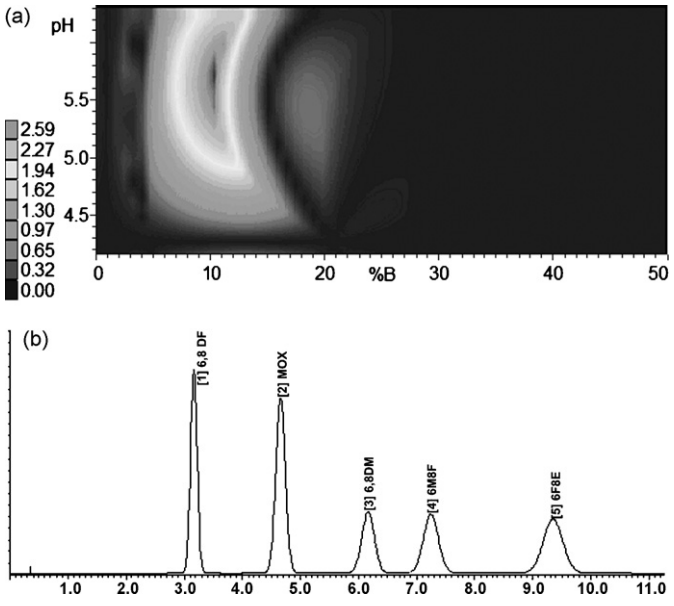


Fig. 4. Isocratic 2D resolution map (a) obtained from gradient data for pH optimization and corresponding chromatogram (b).

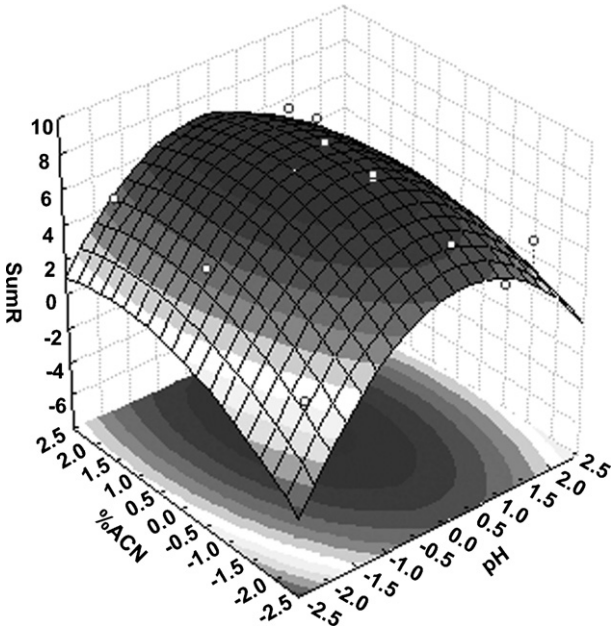


Fig. 5. Response surface calculated from the design data given in Table 3.

Table 3
Experimental design. The overall response factor, R_x was measured by summing the individual resolutions between pairs of peaks.

No.	pH	%ACN	%TEA	R_x
1	0	0	0	9.27
2	0	0	0	9.23
3	0	0	0	9.37
4	0	0	1	8.1
5	0	0	1	8.15
6	0	0	−1	7.47
7	0	0	−1	8.04
8	0	1	0	8.35
9	0	1	0	8.25
10	0	−1	0	8.99
11	0	−1	0	9.26
12	0	−1	−1	7.35
13	0	−1	−1	7.41
14	0	2	−1	5.78
15	0	−1	−1	7.13
16	0	−1	−1	7.12
17	2	2	0	2.38
18	2	−2	0	4.42
19	1.5	0	0	3.5
20	1.5	2	0	5.52
21	1.5	−2	0	2.65
22	1	0	−1	4.45
23	1	0	−1	5.78
24	1	2	−1	6.8
25	0.5	0	−1	6.53
26	0.5	0	−1	7.73
27	0.5	0	−1	6.79
28	0.5	2	−1	5.47
29	0.5	−2	−1	6.34
30	−0.5	0	−1	8.4
31	−1	2	0	3.45
32	−1	−2	0	1.4
33	−1.5	0	0	2.54
34	−1.5	0	−1	3.18
35	−2	0	0	5.18
36	−2	2	0	5.67
37	−2	−2	0	1.48

Table 4
ANOVA analysis of quadratic chromatographic model.

Coefficients	SS	df	MS	*F*	*p*
b_1	0.737	1	0.737	4.508	0.055211
b_{11}	58.553	1	58.553	357.772	0.000000
b_2	4.912	1	4.912	30.014	0.000141
b_{22}	10.207	1	10.207	62.369	0.000004
b_3	0.857	1	0.857	5.239	0.041011
b_{33}	0.852	1	0.852	5.205	0.041571
b_{12}	4.062	1	4.062	24.820	0.000319
b_{23}	0.812	1	0.812	4.961	0.045832
Lack of fit	70.481	16	4.405	26.916	0.000001
Pure error	1.964	12	0.164		
Total SS	198.330	36			

SS: sum of squares, df: degree of freedom, MS: mean sum of squares, *F*: Fisher statistics, *p*: statistical parameter related to significance of coefficients.

Table 5
Calculated coefficients in a quadratic model of chromatographic separation.

Coefficients	Effect	Std. err. pure err.	$t(12)$	*p*
b_0	8.23	0.144	56.9798	0.000000
b_1	0.58	0.272	2.1233	0.055211
b_{11}	−8.90	0.470	−18.9149	0.000000
b_2	1.48	0.270	5.4785	0.000141
b_{22}	−2.80	0.355	−7.8974	0.000004
b_3	0.70	0.306	2.2889	0.041011
b_{33}	−0.91	0.400	−2.2814	0.041571
b_{12}	−1.68	0.337	−4.9819	0.000319
b_{23}	1.03	0.465	2.2273	0.045832

T = 45 °C, %TEA = 2% in close agreement obtained by DryLab® simulation.

2.6. Method validation [25,26]

The optimized chromatographic conditions were validated by evaluating specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and system suitability in accordance with ICH guidelines Q2A.

2.6.1. Linearity

Standard stock solution of the drug and impurities were diluted to prepare linearity standard solutions of impurities in the concentration range 0.2–2.0 μg/ml. Each test solution contained moxifloxacin (200 μg/ml), ofloxacin (1 (g/ml) and one particular impurity standard. Different volumes of stock solutions were transferred into 5 ml volumetric flasks and diluted to mark with the diluent to yield 0.2–2.0 μg/ml concentration range for each impurity. Seven solutions were prepared. The calibration line was obtained by plotting the analyte to IS peak area ratio against corresponding concentration ratio. Three sets of such solutions were prepared and each set was analyzed to plot a calibration curves. The linear coefficients, standard deviation of slope and intercept, correlation coefficient, standard error of the fit, residual sum and standard error in residuals were calculated using the program Statistica v. 6 [22].

2.6.2. Precision, limit of detection, and limit of quantitation

The precision of the HPLC procedure was assessed by analyzing 10 solutions containing known quantities of analytes. The precision was calculated as:

$$\%\text{RSD} = \frac{\text{SD} \times 100}{\bar{x}}$$

The detection limit was determined from calibration curves plotted by using sufficiently low concentrations (0.10, 0.25, 0.35, 0.45, 0.5 and 0.60 μg/ml) of analytes. The limit of detection (LOD) was calculated using the formula:

$$\text{LOD} = 3.3\frac{s_b}{a}$$

where s_b is the standard deviation of *y*-intercept of the calibration line and *a* is the slope of the calibration line. Limit of quantitation (LOQ) was calculated using the equation:

$$\text{LOQ} = 10\frac{s_b}{a}$$

The test solutions at LOD and LOQ concentrations were injected six times and %RSD of peak area of replicate injections was calculated.

2.6.3. Accuracy

Standard mixtures containing MOX, OFLO and four impurities were prepared and analyzed by HPLC using optimal separation conditions. The accuracy of the method was checked for three different impurity concentration levels (relating to nominal one): 80%, 100% and 150%, by standard addition technique. A known amount of impurities was added to the sample containing all components: moxifloxacin, 200.0 μg/ml and ofloxacin as internal standard, 1.0 μg/ml and ratio of peak area (analyte to internal standard) was recorded against added quantity of analyte. All analyses were repeated six times and standard deviations (SD), recoveries and %RSD, were calculated.

2.6.4. Specificity

To demonstrate the specificity of the method the solution of moxifloxacin standard was spiked with known quantities of potential impurities. All the impurities were clearly separated and are

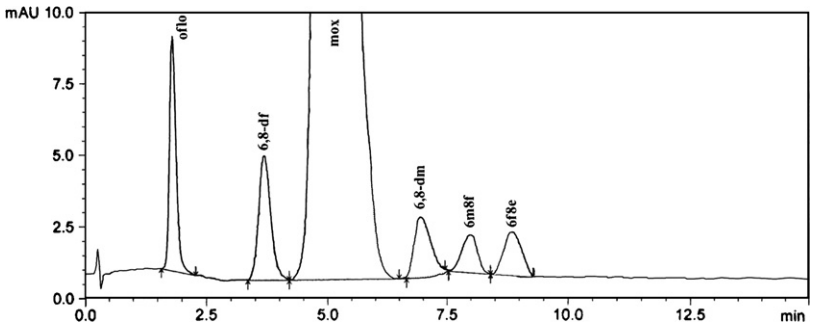


Fig. 6. Chromatogram of ofloxacin (1 μg/ml), moxifloxacin (200 μg/ml) and four synthesis-related impurities (ca. 1 μg/ml) under optimal conditions: pH 6.0; T = 313 K; mobile phase composition (water + 2% triethylamine):acetonitrile = 90:10 (v/v%); flow rate: 1.5 ml/min; λ = 290 nm.

Table 6
Linear regression data for moxifloxacin related impurities.

Compound	Linear regression $Y = aX + b$[a]	Correlation coefficient (R^2)	LOD (μg/ml)	LOQ (μg/ml)	Standard error	Sum of residuals
6,8DF	$Y = (1.06 \pm 0.02)X - (0.04 \pm 0.02)$	0.9988	0.060	0.20	0.02	2×10^{-15}
6,8DM	$Y = (0.75 \pm 0.01)X - (0.14 \pm 0.01)$	0.9994	0.041	0.14	0.01	-7×10^{-16}
6M8F	$Y = (0.86 \pm 0.01)X - (0.14 \pm 0.02)$	0.9990	0.054	0.18	0.02	-6×10^{-16}
6F8E	$Y = (0.99 \pm 0.02)X - (0.12 \pm 0.02)$	0.9986	0.061	0.21	0.02	4×10^{-16}

Concentration range 0.200–2.000 μg/ml. No. of experimental points 7. Internal standard ofloxacin, 1.0 μg/ml.
[a] Y = peak area ratio between analyte and internal standard; X = concentration ratio of analyte and internal standard.

not interfering with the retention times of either moxifloxacin or ofloxacin. A stock solution of placebo was made by dissolving excipients mix (microcrystalline cellulose 136 mg, croscarmelose sodium 32 mg, lactose monohydrate 68 mg, magnesium stearate 6 mg, hypermelosa 10 mg, makrogol 4000 3 mg) in diluent in a 100 ml volumetric flask with sonication and ultrafiltration. Test solutions were made from reference standards and placebo solution.

2.6.5. Robustness

To demonstrate the robustness of the method deliberate small changes of pH and acetonitrile content were made around the optimal values. The pH was varied between 5.8 and 6.2 while acetonitrile content was varied between 9.8 and 11%. No significant changes (relative error less than 5%) of relative retention time (relative to internal standard, ofloxacin) was seen.

2.6.6. System suitability

The system suitability parameters were defined with respect to theoretical plates, tailing factor, repeatability and resolution of the moxifloxacin peak using reference and test solutions.

3. Results and discussion

3.1. Method validation

It was found that the excipients do not interfere with either moxifloxacin or any impurity component. This indicates that the method is specific for the separation and determination of process impurities in moxifloxacin tablets.

A typical chromatogram of a synthetic mixture containing MOX and four impurities: 6,8DF, 6,8DM, 6M8F and 6F8E is shown in Fig. 6. Reproducible peak shapes were obtained under the optimum conditions.

The validation data for calibration lines of four impurities are summarized in Table 6. The response of analytes was linear in the concentration range 0.2–2.0 μg/ml. Calculated statistical parameters indicate that the calibration lines are fitting well into the model and that are significantly linear despite relatively large concentration of moxifloxacin in the test solutions (200 μg/ml). The determined LOD and LOQ values for the impurities indicate their reliable identification and quantification in moxifloxacin pharmaceutical forms. The %RSD of peak area at LOQ concentration was found to be less than 5% indicating satisfactory sensitivity of components quantification.

Using a different HPLC instrument (under the same chromatographic conditions) no significant variation in calculated concentrations, and chromatographic parameters were found (Table 7).

Satisfactory recoveries of the test substances (97.3–102.8%) with %RSD less than 4% were obtained suggesting that method can accurately quantify impurities in both tablets and infusion. Small variations of different parameters (pH, %B) do not result in large change of overall resolution as seen from response surface (Fig. 5) indicating the robustness of the method. To develop a method capable to resolve the drug and its four synthetic impurities in one isocratic run it was necessary to set a minimal base resolution of critical peak pair which should be achieved. We set this value at R_s = 1.4. From DryLab® simulations it was evident that this value can be met in a relatively broad range of ACN percentage in eluent (9–12, v/v%) but a pH range is narrower, between 5.8 and 6.2. Lowering a pH requires much longer analysis time to keep acceptable resolution. In all experiments conducted under optimal conditions, critical reso-

Table 7
Chromatographic characteristics of moxifloxacin and four impurities resolved on a XTerra column under optimal conditions.

Parameter	Compound					
	Instrument	6,8DF	MOX	6,8DM	6M8F	6F8E
RRT	S	0.714	1.00	1.434	1.549	1.710
	A	0.758	1.00	1.540	1.600	1.674
RRF	S	0.991	1.00	0.415	0.404	0.529
	A	1.190	1.00	0.430	0.460	0.589
R_s	S	5.239	2.757	2.779	1.553	1.410
	A	5.460	2.900	2.910	1.600	1.420
Tailing	S	1.189	0.980	1.160	0.976	1.072
	A	1.010	1.000	0.890	0.970	0.984

RRT: relative retention time, RRF: relative response factor, R_s: resolution, calculated relative to ofloxacin as an internal standard, S: Shimadzu, A: Agilent apparatus.

lution was not less than 1.4. Thus, two regions of %*B* and pH overlap at R_s values ranging from minimal 1.4 to as high as 5.5 indicating favorable robustness and suitability of the method. However, tailing of some peaks of impurities deviates from ideal, but nonetheless, chromatograms are reproducible (two instruments) and repeatable. Change in concentration leads to a linear response in peak areas and allows for precise determination. Thus, the experimental tailing is acceptable.

3.2. Impurity determination

An impurity peak at t_R = 8.02 min was noted in tablets and infusion solution. It was well resolved from the standard moxifloxacin (RRT = 1.54). Quantification against moxifloxacin reference standard gives 0.11% of the drug. Comparison of RRT values and DAD spectra with known synthetic impurities indicate that the impurity is 6-methoxy-8-fluoro (6M8F) product. Kumar et al. [12] using preparative gradient HPLC elution separated four process-related impurities from bulk moxifloxacin. The impurity found in our work is different from those found by Kumar et al. The difference can be explained taking into account the fact that in both cases impurities are process related, and we used the samples from Bayer AG while Kumar et al., used the samples from Dr. Reddy's Laboratories Ltd., India. The synthesis and purification procedures employed by these two manufacturers are different, thus, different process-related impurities result.

3.3. Forced degradation products determination

The similar degradation behavior of the drug in bulk, tablets and infusion indicated that there was no interaction between moxifloxacin and excipients. Under hydrolytic conditions two degradation products at t_R = 5.90 and 6.73 min (acid hydrolysis) and t_R = 6.05 and 6.67 min (base hydrolysis) are seen. Total hydrolytic degradation is ca. 0.3%. No peaks of degradants were seen under photolytic or oxidative conditions. Salem et al. [14] found one acid degradant of moxifloxacin by densitometric TLC which was identified by IR spectroscopy as decarboxylated moxifloxacin. Motwani et al. [13] identified by densitometric HPTLC method seven hydrolytic degradates. The difference in degradation behavior of moxifloxacin in our work and that of Motwani et al., can be attributed to much more drastic conditions used for degradation in their work (1 M concentration of HCl or NaOH, 30% H_2O_2, with reflux for 3 h).

4. Conclusion

In the present work a RP-HPLC method for the separation of moxifloxacin impurities was developed with the aid of chemometric approach, validated and used for their determination in tablets and infusion. Optimization based on DryLab® computer simulations and on statistical response surface methodology led to the practically same optimal conditions for separation. The proposed method separates the drug from its process-related impurities and degradants formed during stability testing in a reasonable analysis time and with acceptable chromatographic parameters.

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