Micellar Liquid Chromatographic Method for Determination of Moxifloxacin and its Impurities

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Abstract

A selective eco-friendly micellar HPLC method was developed for investigation of moxifloxacin and related compounds in the presence of its degradation products. Central composite design was used to optimize the experimental conditions. The proposed method is based on isocratic elution on a C18 column using 92.5% (v/v) biodegradable aqueous mobile phase containing 0.01 M sodium dihydrogen phosphate, 0.15 M sodium dodecyl sulfate (SDS) and 0.5% triethylamine (v/v) with a pH of 3.5 and 7.5% isopropanol (v/v) as eco- friendly organic solvent. The flow rate and injection volume were 0.6 ml/min and 5 μl, respectively. Experiments were performed at a temperature of 60 °C and detection was performed at 295 nm. The optimized method was validated. The method was found to be suitable for the quantification of moxifloxacin and its related compounds in moxifloxacin drug substance. The Green Analytical Procedure Index (GAPI) proves the superiority of the developed method against other reported methods.

Keywords: Moxifloxacin, Micellar, Impurities, SDS, Degradation, GAPI

Introduction

Moxifloxacin is an anti-infective from the group of fluoroquinolones. Moxifloxacin drug substance is described in the European (Ph. Eur.) and United States Pharmacopeia (USP).[1–2] High-performance liquid chromatography (HPLC) is the most commonly used analytical tool for pharmaceutical analysis. Most published HPLC methods for investigation of moxifloxacin are based on the reversed-phase (RP) mode using organic solvents such as acetonitrile and methanol in mobile phase.

These two solvents are not preferred in terms of environmental impact and health safety. Even if methanol is less toxic and more easily biodegradable than acetonitrile, it is also ranked as a hazardous solvent due to its inherent toxicity and the great requirements of its waste disposal.

The organic solvents commonly accepted as green, and which can be used in RP-HPLC, are ethanol, isopropanol, n-propanol, acetone, ethyl acetate, ethyl lactate, and propylene carbonate. [3]

The official HPLC methods for the analysis of the drug substance moxifloxacin and its impurities described in Ph. Eur. and USP are essentially similar and use a mobile phase containing methanol and aqueous solution containing 0.5 g/l tetrabutylammonium hydrogen sulfate, 1 g/l potassium dihydrogen phosphate and 3.4 g/l phosphoric acid (28:72, v/v).

Also, most reported HPLC methods for determination of moxifloxacin and its impurities use a high percentage of organic solvents (methanol or acetonitrile) in the mobile phase which cannot be considered as environmentally friendly solvents. [4–11]

Micellar liquid chromatography (MLC) method has been investigated as an interesting approach for green analytical chemistry (GAC), as it eliminates or reduces the use of organic solvents and uses mobile phases containing 90% (v/v) or more water.[12–17] MLC is attractive due to its lower cost, lower toxicity, greater stability, reduced negative impact on the environment and greater safety for laboratory use. MLC depends on using surfactants at a concentration above their critical micellar concentration (CMC).[13]

The choice of surfactant is of great importance when creating a hybrid micellar chromatography system. In previous research, sodium dodecyl sulfate (SDS) was most often examined with regard to its availability and low price.[18] The large amount of data available in the literature on mi-

cellar and hybrid micellar systems with SDS as a surfactant facilitates the setting up of chromatographic methods for specific analyses.[19–23]

Another important advantage of MLC concerns sample treatment. In fact, the great solubilizing ability of micelles allows the direct injection of drugs in complex matrices (e.g., biological fluids and dosage forms) without the need for any sample pretreatment other than filtration.[3] Moreover, MLC is compatible with existing RP-HPLC instruments. Therefore, it does not require any modification of existing RP-HPLC instrumentation.[3]

To the best of our knowledge, no method has been reported for the determination of moxifloxacin and its related substances by micellar HPLC. A few papers have been published on the topic of determination of fluoro-quinolone using micellar HPLC.[24–25]

In a published study[24] a method for simultanous separation of four quinolones including moxifloxacin was developed. Also, a study was conducted on the simultaneous separation of levofloxacin and ambroxol.[25] The aim of this work was to develop an environmentally friendly MCL method for the investigation of moxifloxacin and related compounds (Figure 1) in the presence of its degradation products, using ecologically safer mobile phase composition and lower solvent consumption.

The proposed method was compared favorably with published methods using the new assessment tool, GAPI index, to provide additionally support for the environmental benefits of the proposed method.

2. Experimental

2. 1. Materials

A moxifloxacin drug substance sample was provided by the Hetero Drugs Limited, India. Moxifloxacin hydrochloride CRS (purity of 96.1%) was purchased from EDQM. Five moxifloxacin impurities namely Impurity-A, Impurity-B, Impurity-C, Impurity-D, Impurity-E (Figure 1) with stated purity of 99.94%, 100.00%, 99.72%, 99.75%, 98.82%, respectively, were purchased from Veeprho Pharmaceuticals s.r.o, Europe. All reagents used were of analytical grade.

Sodium phosphate monobasic monohydrate was purchesed from CARLO ERBA (CARLO ERBA Reagents S.A.S, France), SDS was purchased from ACROS OR-GANICS (ACROS ORGANICS, Geel, Belgium), triethylamine and orthophosphoric acid were supplied by Fischer Scientific U.K. Limited.

For the mobile phase, isopropanol was HPLC grade purchased from Fischer Scientific U.K. Limited. HPLC grade water was produced using a Milli-Q purification system (Millipore Co., MA, USA) provided by ZADA Pharmaceuticals.

2. 2. Equipment and Chromatographic Conditions

Chromatographic analyses were done using a *Thermo Finigian* HPLC system (*Thermo Fisher Scientific Inc.*, Waltham, SAD) equipped with a DAD detector. Separations were achieved on a ZORBAX SB C18 150 mm x 4.6
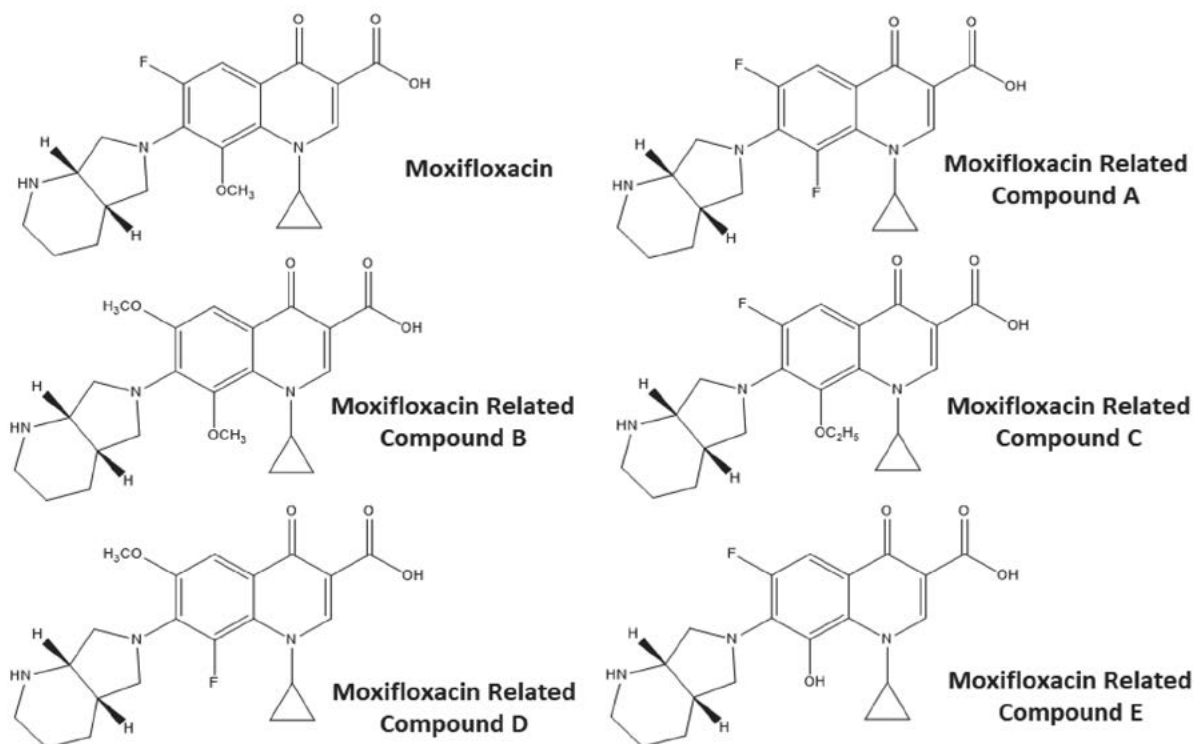

Figure 1. Structures of moxifloxacin and its known related compounds

mm; 3.5 μm particle size column. Aqueous part of the mobile phase was prepared using 0.01 M sodium dihydrogen phosphate, with added SDS (0.15 M) and triethylamine (0.5%, v/v). The pH was adjusted to 3.5 with orthophosphoric acid HPLC electrochemical grade, after adding SDS and other components. Lastly, 92.5% (v/v) of aqueous phase was mixed with 7.5% of isopropanol (v/v).

The flow rate and injection volume were 0.6 ml/min and 5 μl, respectively. Experiments were performed at a temperature of 60 °C and detection was performed at 295 nm. Before use, the mobile phase, standard and sample solutions were filtered through a 0.45 μm nylon filter (*LLG Labware*, Meckenheim, Germany).

2. 3. Standard and Samples Solution

Standard solution for determination of moxifloxacin assay was prepared using mobile phase as diluent in concentration of 0.1 mg/ml. Standard solutions for determination of moxifloxacin impurities in concentration of 0.2 μg/ml using the same solvent were also prepared. Moxifloxacin drug substance sample solution was prepared in a concentration of 0.1 mg/ml.

2. 4. Stress Samples

To conduct the forced degradation study, moxifloxacin was subjected to acidic, alkaline, oxidative, thermal, UV light, humidity and photolytic conditions. Stress samples for acid and base hydrolysis were prepared using 4 M HCl and 4 M NaOH as stress agents. Sample stock solution of moxifloxacin drug substance was prepared in concentration of 1 mg/ml using the mobile phase as a diluent. 1 ml of stock solution was transferred to 10 ml volumetric flasks, then 1 ml of each different stress agent was added to each volumetric flask containing the stock solution. The solutions containing an acidic stress agent were subjected to a temperature of 70 °C and the solutions containing the base were subjected to a temperature of 50 °C, in each case for a period of 6 days. After the stress treatment, samples were diluted up to the volume of the 10 ml flask with the same diluent (0.1 mg/ml concentration).

For degradation under oxidizing conditions, the drug was heated under reflux with 3 % H_2O_2 (v/v) at room temperature for 24 hours. For thermal degradation, the powdered drug was exposed to a temperature of 70 °C for 48 hours. With respect to photodegradation, powdered moxifloxacin was exposed to UV light for 3 days and daylight for 7 days. Within the stress studies, untreated, zero time and blank samples were prepared as controls in addition to stress samples.

2. 5. Method Optimization

Central composite design was used to optimize the experimental conditions in order to develop an appro-

priate method with the shortest possible run time and maximum resolution factors for the critical peaks. During the optimization, the effects of column temperature (A), amount of organic solvent (B), pH (C) and flow rate of a mobile phase (D) were studied. Resolution factors for the critical peaks and retention time of the last eluted component were selected as observed responses.

For method optimization, a sample was prepared by adding five known moxifloxacin impurities (impurities A, B, C, D and E) to the stress sample.

Central composite design was performed using *Design-Expert 7.0* (*Stat-Ease Inc.*, Minneapolis, SAD). Thirty experiments were conducted with the aim of investigating the influence of four variables (A, B, C and D). The plan of the experiment and observed responses are presented in Table 3.

2. 6. Method Validation

System suitability test (SST)

SST for moxifloxacin assay determination was performed by six replicate injections of moxifloxacin standard solution in concentration of 0.1 mg/ml. The parameters evaluated included relative standard deviation (% RSD) for peak area, tailing factor, and column efficiency.

SST for determination of related substances was evaluated including resolution factors for the critical peaks and % RSD for peak area for each known moxifloxacin impurity.

To evaluate the resolution factors for the critical peaks, sample containing moxifloxacin hydrochloride in concentration of 0.1 mg/ml with added five known moxifloxacin impurities in concentration of 0.2 μg/ was injected. Six replicate injections of a sample which containing five known moxifloxacin impurities and moxifloxacin CRS in concentration of 0.2 μg/ml were injected to evaluate % RSD for peak area of impurities.

Selectivity test

The selectivity of the optimized MLC method was evaluated by comparing chromatograms obtained from the analysis of solvent, sample solution, sample solution with known impurities, stress sample solution and stress agents sample solution. The method was considered selective if the peaks observed in the chromatograms were well separated and there were no co-eluting peaks at retention time of moxifloxacin, known moxifloxacin impurities and degradation products from forced degradation studies. The acceptance criteria applied was related to the resolution factor calculated for adjacent peaks of all analytes and which had to be equal to or greater than 1.5. In addition, a diode array detector (DAD) was used to evaluate peak purity based on the peak purity index.

Linearity

The linearity of the optimized MLC method was evaluated by fitting the calibration data using least squares

regression with five different concentrations of moxifloxacin in the range of 0.05–0.15 mg/ml (50–150% of the target concentration denoted as 0.1 mg/ml) and five different concentrations for each known moxifloxacin impurity in the range of 0.1–0.3 μg/ml (50–150% of the target concentration 0.2 μg/ml, selected considering the specification limit for moxifloxacin related substances defined in Ph.Eur. and USP). Linearity was evaluated by the values of the correlation coefficient (R^2 value).

Accuracy

The accuracy of the method was determined by analyzing a solution containing moxifloxacin and all impurities at three different concentrations (80%, 100% and 120% with respect to the target value) of each in triplicate at the specified limit. The percentage of recoveries for each analyte was calculated by injecting the standard solution for each level.

Precision

The inter-day precision of the method was checked by injecting six invidual solutions containing moxifloxacin and its impurities in target concentrations of 0.1 mg/ml and 0.2 μg/ml, respectively. The % RSD for the peak area of each analyte was calculated. The intermediate precision of the method was also evaluated using different analyst and different instruments in the same laboratory using appropriate sets of solutions prepared in the same way as in case of inter-day precision.

Robustness

The robustness of the method was investigated by analysing the results of previously performed central composite design. The robustness was evaluated considering the same factors as used for method optimization: column temperature (A), amount of organic solvent (B), pH (C) and flow rate of a mobile phase (D).

Limit of detection and Limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined experimentally by measuring the signal-to-noise ratio of the each substance by injecting a series of dilute solutions with known concentration. LOD and LOQ were determined with 3.3s/n and 10s/n criteria, respectively from the data from calibration curve.

3. Results and Disscusion

3. 1. Development of MLC Method

Preliminary studies were performed to select an efficient method for the analysis of moxifloxacin and related substances in the presence of its degradation products. The aim was to apply an eco-friendly MCL method with isocratic elution. In the RP-HPLC system, special atten-

tion is paid to the selection of the stationary phase. The most commonly used stationary phases are silica stationary phases modified with alkyl groups, such as C18 and C8. However, in micellar chromatography systems, in addition to the selection of a suitable stationary phase, the choice of surfactant is also very important. The presence of a surfactant significantly changes the properties of the stationary phase and opens up possibilities for numerous and varied interactions with analytes.[26]

Development began with the use of anionic SDS as one of the most commonly used surfactant in MLC. A solution of sodium dihydrogen phosphate with the addition of SDS and triethylamine was used as aqueous solvent, and isopropanol was used as organic solvent.

A C18 HPLC column was used. The preliminary tests were initially necessary to determine whether the proposed method could separate the peak of moxifloxacin and the peaks of moxifloxacin impurities defined by the pharmacopoeia (impurities A, B, C, D and E). [1,2]

Preliminary tests were based on the identification of the main factors that could affect the separation of analytes: pH of the mobile phase, possibility of using different water phases (buffer solution), concentrations of SDS and triethylamine, percentage of organic solvent and possibility of using different organic solvents, influence of column temperature and flow rate.

According to the values of pKa and log P as a function of pH, the pH of the mobile phase was selected to be pH=3.0. The pKa values and pH dependence on the ionic and non-ionic forms of the substances indicated that all analytes are present in the protonated form on the N-heterocycle of piperidine, as the NH+ and -COOH group are non-ionized, which ensures interactions with the negatively charged surface of the surfactant adsorbed on the stationary phase and the negatively charged surface of the micelle.

0.01 M sodium dihydrogen phosphate was used as a basic aqueous solution to have efficient control of the pH of the mobile phase.

Solubilizing capacity of micelles and consequently their influence on retention was analysed by varying the concentration of SDS (0.10 M and 0.15 M). It was found that there was no significant difference in the quality of chromatographic separation of all adjacent chromatographic peaks with different SDS concentration. Increased concentration of SDS (0.15 M) resulted in a shorter run compared to the lower concentration of SDS (0.10 M), which is explained by the fact that the surfactant increases the affinity and interactions with the surfactant monomers in the mobile phase, resulting in a decrease in retention. Reduction in the retention time of the analyte is usually achieved by increasing the concentration of the surfactant or the organic solvent.[27] In order to shorten the time required for the analysis, the SDS concentration of 0.15 M was chosen. Concentration of SDS (0.15 M) in our micellar system is higher than the CMC. Increasing the surfactant concentration above the CMC does not affect the critical

micelle concentration because any added monomer is incorporated into the micelles, while the concentration of free surfactant monomers does not change and remains equal to the CMC.[28]

Variations in the percentage of triethylamine (0.5%, 0.7% and 1.0%, v/v) were also investigated. This additive usually contributes to the symmetry of the chromatographic peaks by minimizing the undesirable secondary interactions of the basic analytes with free silanol groups on the surface of the stationary phase.[29] The addition of small amounts, 0.1–0.2% (v/v), of triethylamine in chromatography is typically used to reduce the effect of tailing for basic small molecule compounds.[29] In this regard, changing the peak shape may affect the base line separation of closely spaced elution peaks. When the percentage of triethylamine is increased, co-elution of the impurity A peak with the moxifloxacin peak occurs, disturbing the purity of the peak, while with 1.0% triethylamine the separation of impurity A and moxifloxacin peak is not achieved. The best conditions were achieved with 0.5% (v/v) triethylamine. This concentration of triethylamine can affect the decrease of the critical micelle concentration since the amount of triethylamine added is larger than would be added as a typical stationary phase modifier, a large portion of the triethylammonium ion remains in the mobile phase. Although the triethylammonium ion and negatively charged SDS sulfate monomers will not spontaneously form dodecyltriethylammonium sulfate, some electrostatic attraction may occur between the two which could further inhibit the monomers from adsorbing to the stationary phase.[29]

In order to increase the efficiency of the MCL method and achieve retention in a suitable time, it was necessary to add an appropriate amount of organic solvent to the aqueous mobile phase. The use of the most commonly used organic solvents in MCL, isopropanol and acetonitrile, was considered. Since it is well known that the organic solvent increases the affinity and interactions of the analyte with the mobile phase, and desorbs SDS from the stationary phase, it was necessary to determine the optimal amount of the organic solvent.[30] The addition of propanol to the aqueous micellar solution of SDS leads to a decrease in CMC. [31] The tests were based on the use of isopropanol and the variation of its percentage, but the possibility of using acetonitrile (10%, 15% and 20% (v/v) in the mobile phase) was also tested. Acetonitrile did not prove to be the solvent of choice, since its use did not separate all known impurities. It is also very important to avoid the use of acetonitrile, since the aim of the method was to use a less toxic organic solvent such as isopropanol.

The percentage of isopropanol in the mobile phase was varied between 2.5% and 8.0% (v/v). In preliminary tests, satisfactory chromatographic conditions and system responses were achieved with 5.0% (v/v) isopropanol.

The influence of column temperature was also investigated. Since the most frequently reported cases in the literature were analyzes performed at a column temperature of 25 °C, the experiments were initially performed at this temperature. An increase in temperature can affect the micellization process and increase the CMC because it destroys the ordered structure of water around the hydrophobic surfactant groups so that the micelles are broken down.[31] Unfortunately, satisfactory chromatographic separations could not be obtained at the temperature of 25 °C and the column temperature was carefully increased to 50 °C. The choice of column temperature of 50 °C showed
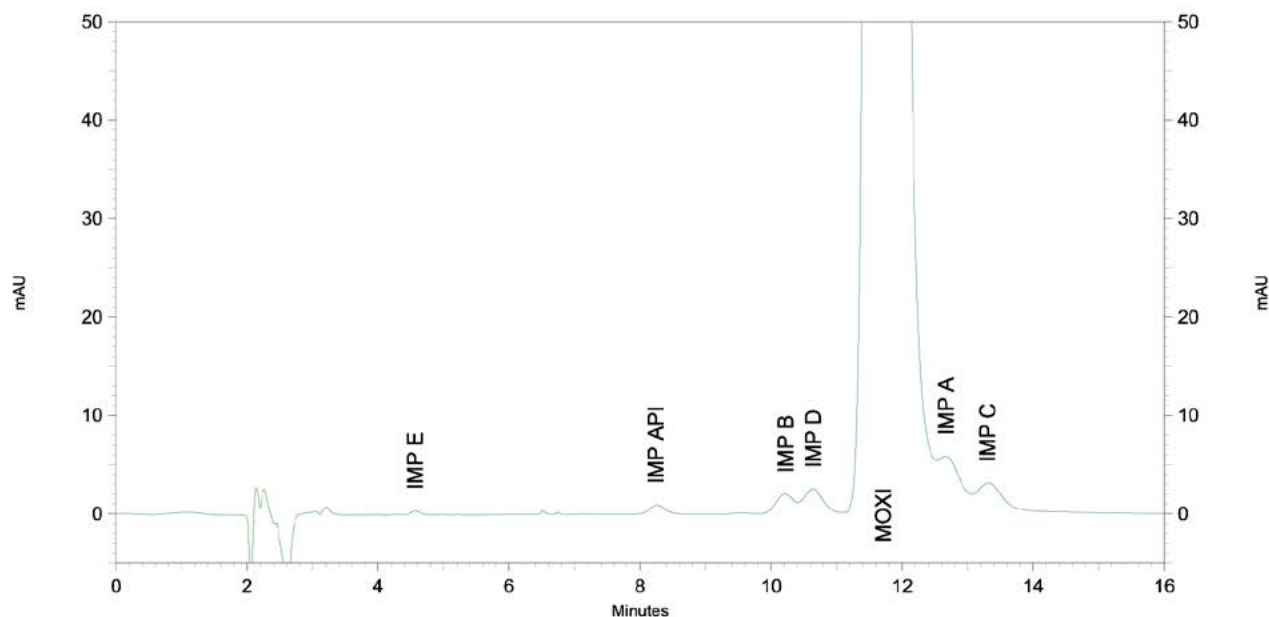

Figure 2. Sample of moxifloxacin and its known related compounds

that an unexpected phenomenon occurred, namely the increased effect of temperature on the reduction of hydration of the polar groups, which further favored.[32]

During the preliminary tests, the experiments were started with a flow rate of 1.0 ml/min. However, despite simultaneous variation of other chromatographic conditions with this flow rate, it was not possible to separate impurity A from moxifloxacin because impurity A peak eluted immediately after the moxifloxacin peak. Accordingly, the flow rates of 0.8 ml/min and 0.5 ml/min were tested. Finally, with the reduction of the flow rate to 0.5

ml/min, the separation of the critical peaks was achieved (Figure 2).

3. 2. Forced Degradation Study

In forced degradation studies, moxifloxacin drug substance was found to be extremely stable to: thermal and photodegradation, oxidative stress and base hydrolysis. Significant degradation was caused by acidic conditions, using very high concentration of acid (4M HCl) and at extremely high temperature (Figure 3, Table 1)
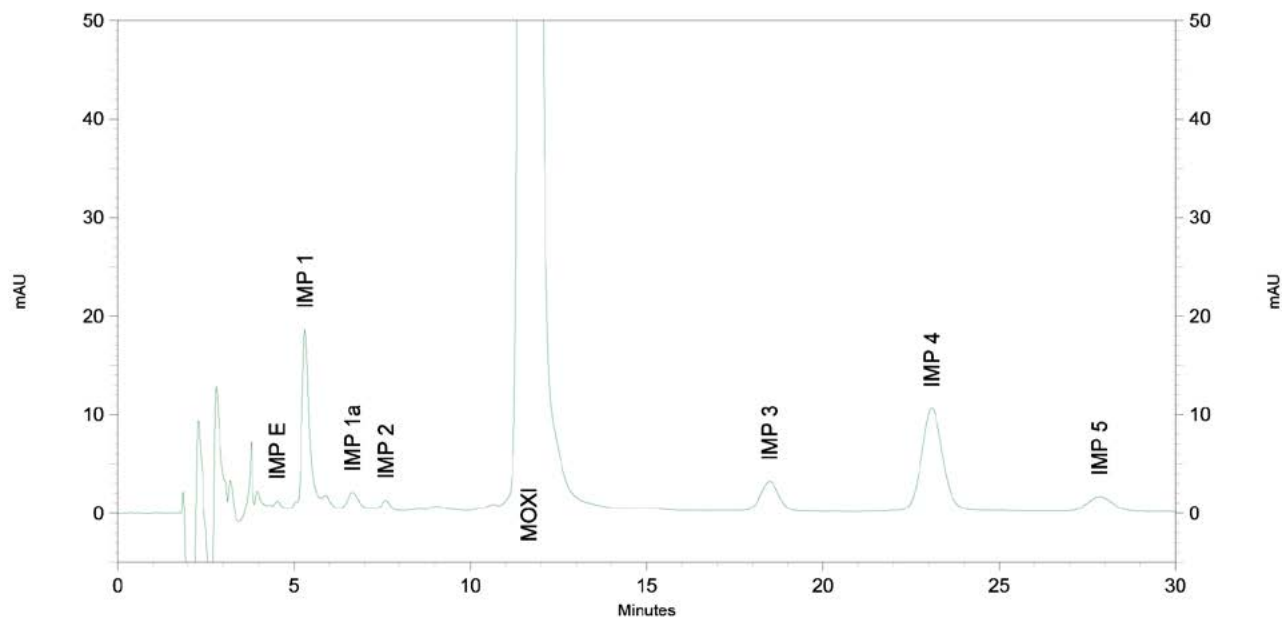

Figure 3. Stress sample of moxifloxacin subjected to acidic hydrolysis

Table 1. Results of forced degradation studies

Stress condition	Moxifloxacin	Percentage of degradation (%)	Degradation products (content %)	Moxifloxacin peak purity content (%)
Untreated sample	101.20	–	–	0.999940
Acid hydrolysis (4 mol/l HCl, 70 °C, 6 days)	85.19	16.01	IMP E (0.018%) IMP 1 (0.841%) IMP 1a (0.112%) IMP 2 (0.049%) IMP 3 (0.351%) IMP 4 (1.469%) IMP 5 (0.239%)	0.999845
Base hydrolysis (4 mol/l NaOH, 50 °C, 6 days)	100.41	–	–	0.999899
Oxidation (3 % H$_2$O$_2$, room temperature, 24 hours)	100.20	–	–	0.999887
Photodegradation (UV 254 3 days, daylight 7 days)	101.05	–	–	0.999989
Thermal degradation (70 °C, 2 days)	100.95	–	–	0.9999978

for 6 days. The strength of the stress agents was chosen in such a way that the degradation was in the range of 5–20%. Preparation of three additional control samples was mandatory for the correct determination the obtained results. The published study also demonstrated that moxifloxacin is very stable under all conditions recommended by ICH Q1A (R2) at lower concentration of acid and base.[33]

The percentage of degradation and the mass balance on all tested samples were calculated (Table 1) and the content of all degradation products formed was reported .

Forced degradation studies were performed for moxifloxacin drug substance to provide an indication of the specificity of the proposed method.

3. 3. Optimization

Central composite design was performed using 30 experimental runs. The levels of independent variables namely column temperature (A), amount of organic sol-

vent (B), pH (C) and mobile phase flow rate (D), and the responses or dependent variables are shown in Table 3. According to the preliminary results independent variables were tested on the level presented in Table 2.

Table 2: Level of investigated variables

Variables	Level		
	(−1)	(0)	(+1)
Column temperature (°C, A)	50	55	60
Amount of isopropanol (%, B)	2.5	5.0	7.5
pH (C)	2.5	3.0	3.5
Flow rate (ml/min, D)	0.4	0.5	0.6

Optimization study was done using stress sample (acid hydrolysis) spiked with five known moxifloxacin impurities (impurities A, B, C, D and E).

Statistical parameters for the selection of the best fit model (p value <0.05; Lack of Fit >0.05, R^2 value >0.8) were achieved for all five responses (Table 4).

Table 3. Central composite design results using four independent variables

Run	Variables				Responses				
	Column temperature (°C; A)	Amount of organic solvent, isopropanol (%; B)	pH (C)	Flow rate (ml/min; D)	Resolution (IMP B / IMP D)	Resolution (IMP D/MOXI)	Resolution (MOXI /IMP A)	Resolution (IMP A/ IMP C)	Retention time $_{IMP\,5}$
1	50	2.5	2.5	0.4	1.16	3.15	2.15	1.22	44.48
2	60	2.5	2.5	0.4	1.16	2.97	2.24	1.31	42.35
3	50	7.5	2.5	0.4	1.04	1.70	1.73	2.21	36.27
4	60	7.5	2.5	0.4	1.10	1.76	1.73	2.22	34.20
5	50	2.5	3.5	0.4	0.96	2.87	2.29	1.05	30.88
6	60	2.5	3.5	0.4	1.10	2.91	2.35	1.05	28.58
7	50	7.5	3.5	0.4	1.14	1.71	2.10	2.21	27.60
8	60	7.5	3.5	0.4	1.27	1.64	2.10	2.11	25.35
9	50	2.5	2.5	0.6	1.01	2.85	2.16	1.41	29.42
10	60	2.5	2.5	0.6	1.26	3.07	2.10	1.51	27.78
11	50	7.5	2.5	0.6	1.05	1.67	1.53	2.21	23.90
12	60	7.5	2.5	0.6	1.10	1.70	1.50	2.20	22.30
13	50	2.5	3.5	0.6	0.81	2.73	2.15	1.21	21.28
14	60	2.5	3.5	0.6	1.08	2.61	2.14	1.25	19.46
15	50	7.5	3.5	0.6	1.07	1.66	1.73	2.05	20.63
16	60	7.5	3.5	0.6	1.20	1.67	1.78	2.05	18.81
17	50	5.0	3.0	0.5	0.89	2.28	1.81	1.60	27.08
18	60	5.0	3.0	0.5	0.98	2.21	1.90	1.60	25.16
19	55	2.5	3.0	0.5	1.00	2.56	2.07	1.30	28.08
20	55	7.5	3.0	0.5	1.14	1.59	1.54	2.20	23.65
21	55	5.0	2.5	0.5	1.51	2.56	1.50	1.78	31.00
22	55	5.0	3.5	0.5	1.50	2.19	1.82	1.54	22.53
23	55	5.0	3.0	0.4	1.08	2.53	1.94	1.60	32.71
24	55	5.0	3.0	0.6	1.05	1.67	1.94	1.60	21.89
25	55	5.0	3.0	0.5	1.18	2.57	1.75	1.61	25.92
26	55	5.0	3.0	0.5	1.04	2.33	1.79	1.58	25.90
27	55	5.0	3.0	0.5	0.93	2.43	1.80	1.58	25.93
28	55	5.0	3.0	0.5	1.03	2.39	1.76	1.60	25.92
29	55	5.0	3.0	0.5	0.97	2.33	1.83	1.57	25.90
30	55	5.0	3.0	0.5	1.02	2.37	1.83	1.61	25.91

Table 4: Statistical parameters for selection of the best fit model

	$R_{s(IMP\ B\ /\ IMP\ D)}$	$R_{s\ (IMP\ D/\ MOXI)}$	$R_{s(MOXI/\ IMP\ A)}$	$R_{s(IMP\ A/\ IMP\ C)}$	Run time IMP 5
Model	quadratic	linear	quadratic	quadratic	quadratic
p-value	0.0005	<0.0001	<0.0001	<0.0001	<0.0001
Lack of fit	0.6316	0.0578	0.0820	0.0562	0.083
R^2	0.8547	0.8995	0.9710	0.9963	1.000
R^2adjusted	0.7191	0.8687	0.9439	0.9929	1.000

The proposed models for five observed responses were as presented in following equations (1-5):

$$Rs_{Imp\ B/Imp\ D} = 1{,}08 + 0{,}062A + 0{,}032B - 0{,}014C - 0{,}021D\ 0{,}018AB + 0{,}019AC + 0{,}023AD + 0{,}064BC + 5{,}625*10^{-3}BD - 0{,}017CD - 0{,}20A^2 - 0{,}069B^2 + 0{,}37C^2 - 0{,}074D^2 \quad (1)$$

$$Rs_{Imp\ D/Moxi} = 2{,}29 - 4{,}444*10^{-3}A - 0{,}59 B - 0{,}080\ C - 0{,}089\ D\ (Eq.2) \quad (2)$$

$$Rs_{Moxi/Imp\ A} = 1{,}78 + 0{,}011A - 0{,}22B + 0{,}10C - 0{,}089D - 3{,}750*10^{-3}AB + 6{,}250*10^{-3}AC - 0{,}013AD + 0{,}059\ BC - 0{,}040\ BD - 0{,}030\ CD + 0{,}091A^2 - 0{,}041B^2 - 0{,}10C^2 + 0{,}18D^2 \quad (3)$$

$$Rs_{Imp\ A/Imp\ C} = 1{,}61 + 7{,}222*10^{-3}A + 0{,}45B - 0{,}086C + 0{,}028D - 0{,}021AB - 0{,}016AC + 8{,}125*10^{-3}AD + 0{,}029\ BC - 0{,}062\ BD - 0{,}014\ CD - 0{,}028A^2 + 0{,}12B^2 + 0{,}032C^2 - 0{,}028D^2 \quad (4)$$

$$k_{Imp5} = 25{,}91 - 0{,}98A - 2{,}20B - 4{,}25C - 5{,}39D + 9{,}379*10^{-3}AB - 0{,}047AC + 0{,}12AD + 1{,}22BC + 0{,}66BD + 1{,}35CD + 0{,}21A^2 - 0{,}043B^2 + 0{,}86C^2 + 1{,}39D^2 \quad (5)$$

The *Desirability* function evaluation was introduced with the aim to make compromising solution that satisfies following optimisation objectives: the least possible run time and the maximum resolution factors for the critical peak pairs. The analysis of the 3D chart presented in Figure 4 enabled the definition of the experimental region for which the *Desirability* function is equal to 1 indicating the maximal fulfillment of all predefined optimization objectives. The observed responses under optimized chromatographic conditions, were as presented in Table 5.

The chromatographic conditions finally selected were pH 3.5 and 7.5% isopropanol (v/v) in the mobile phase, flow rate of 0.6 ml/min and column temperature of 60 °C. According to these optimal chromatographic conditions, representative chromatogram was recorded and presented on the Figure 5.
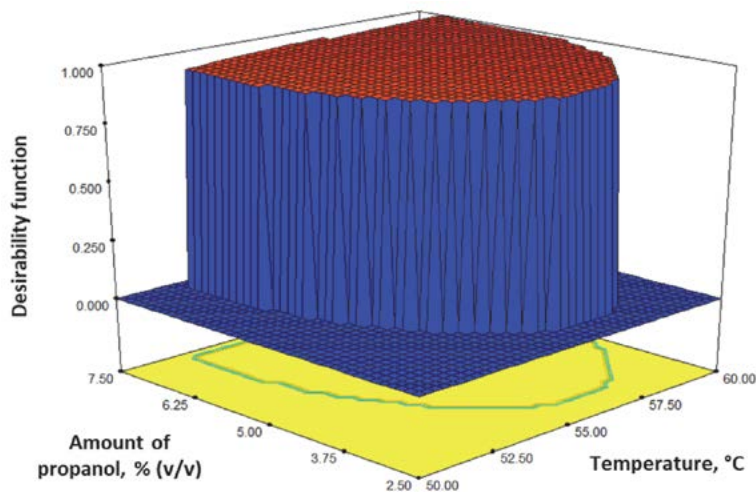
Figure 4. Response surface plot of desirability function

Table 5. Responses of the system under optimal experimental conditions

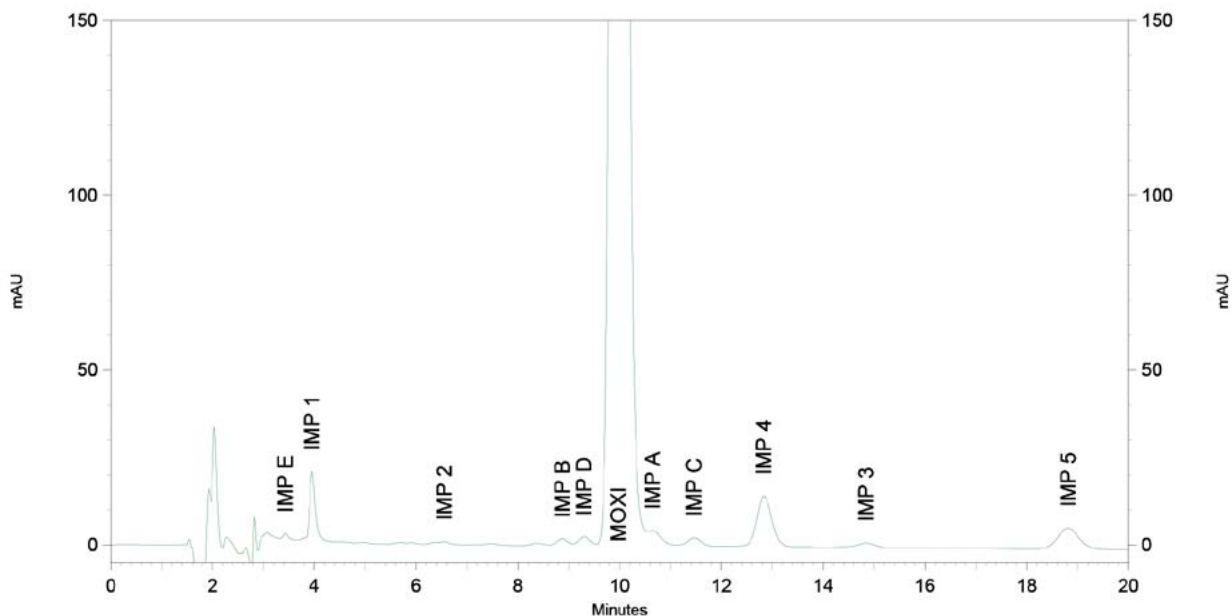| Resolution factors for critical peaks (>1.50)				Run time
$R_{s\ (IMP\ B\ /\ IMP\ D)}$	$R_{s\ (IMP\ D/\ MOXI)}$	$R_{s\ (MOXI/\ IMP\ A)}$	$R_{s\ (IMP\ A/\ IMP\ C)}$	
1.20 (p/V=5.5) RSD <5%	1.67	1.78	2.05	18.81

Figure 5. Stress sample of moxifloxacin under acid hydrolysis with added known moxifloxacin impurities (under optimal chromatographic conditions)

3. 4. Validation

The optimized method was validated and met all acceptance criteria required by ICH regulation.[34] Summary of the validation results is presented in Table 6. Before validation of the HPLC method, SST was performed. SST parameters evaluated for the determination of moxifloxacin assay were RSD for peak area (<1%), tailing factor (<2%), and the number of theoretical plates (>2000) while SST parameters for determination of moxifloxacin impurities were resolution between critical peaks (>1.5) and % RSD for peak area for each known impurity (<5%).

Selectivity test showed stability indicating property and specificity of the proposed method peak purity index was between 990 and 1000 showing that there were no co-eluting peaks with moxifloxacin, moxifloxacin known impurities and degradation products. In addition, resolution values of the analytes (moxifloxacin and impurities) were >1.5.

Linearity test showed that there was an excellent correlation between the peak area and concentration of moxi-

floxacin and all five impurities. All calibration curves were linear (R^2 >0.99) over the calibration ranges tested. The ranges were 50%–150% of the specification limit of each tested analyte (Table 6).

Accuracy of moxifloxacin and all five impurities was found to be in between the predefined acceptance criteria of 80% to 120% and the data given in Table 6.

The Precision was determined at the concentration of 0.2 µg/ml for all impurities and 0.1 mg/ml for moxifloxacin and the % RSD was found to be below 5% for all impurities and below 2% for moxifloxacin (Table 6).

The robustness of the method was investigated by experimental design methodology using the same considerations of the effects of column temperature (A), amount of organic solvent (B), pH (C) and flow rate of a mobile phase (D). Analyses of 3D response surfaces plotted using equations (1)-(5) were done taking in mind the usual variation of experimental factors in the ranges required for robustness testing which arise from analytical measurement uncertainty (e.g. column temperature in the range ± 5 °C

Table 6. Validation results summary

|	Moxifloxacin	Impurity A	Impurity B	Impurity C	Impurity D	Impurity E
R^2 value concentration range 0.05–0.15 mg/ml (for moxifloxacin) 0.1–0.3 µg/ml (for impurities)	0.99895	0.99867	0.99669	0.99976	0.99483	0.99935
Accuracy at 80%	98.80	104.41	106.29	104.99	105.95	100.69
Accuracy at 100%	97.29	97.89	101.07	98.17	101.03	97.40
Accuracy at 120%	101.26	97.70	94.32	92.31	92.24	90.99
Precision (% RSD)	1.38	2.98	3.38	3.17	3.58	2.51
Limit of detection (ng/ml)	5.2	11.2	18.2	5.3	11.8	8.0
Limit of quantification (ng/ml)	15.8	34.2	55.3	16.2	35.7	24.3
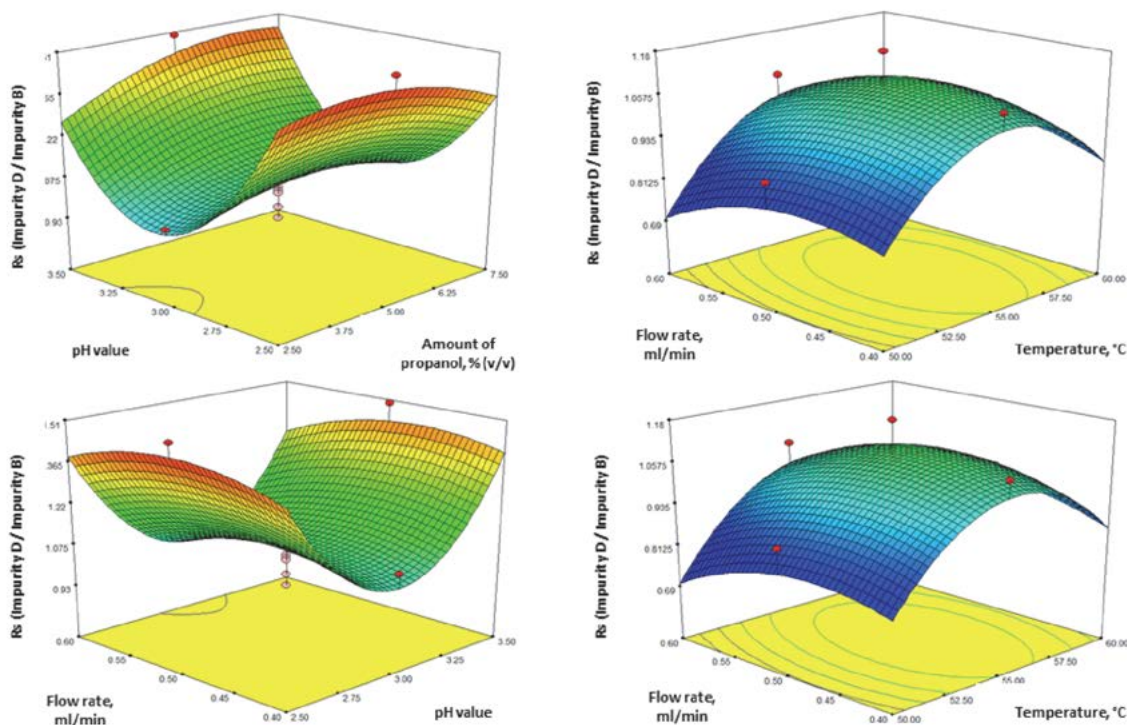
Figure 6. Response surfaces and estimated contours of the resolution of critical peak pair

from the nominal value set within method optimization). It was considered that the moderate slope of the 3D response surfaces related to the particular influence should indicate that the method is robust towards this experiment. The curvature of the 3D response surface indicates the presence of factor interactions and it was interpreted in combination with the slope. According to the representative 3D response surfaces presented in Figure 6 it can be concluded that careful maintenance of method settings is very important in order to retain the satisfactory chromatographic behavior of demanding mixture of analytes as the one used in this study. Fortunately, the common instrument qualification procedure should result in proper control of instrument as well as other experimental factor variations.

3D responses showed that the method has acceptable robustness under the given controlled conditions.

Limit of detection and Limit of quantification for known impurities were in ranges 5.3–18.2 ng/ml and 16.2–55.3 ng/ml, respectively. LOD and LOQ values for moxifloxacin were 5.2 ng/ml and 15.8 ng/ml, respectively.

3. 5. Comparison with Other Reported Methods

Most published methods for investigation of moxifloxacin and its impurities, including the official Ph. Eur. and USP methods, are based on the RP-HPLC mode using organic solvents, such as methanol and acetonitrile in the mobile phase, which cannot be considered as environmentally friendly solvents.

The proposed MCL method has advantages in term of greenness since it uses mixture of biodegradable aqueous mobile phase containing SDS and a much lower percentage of the more eco-friendly organic solvent, isopropanol, compared to the other published methods.

As for greenness, several tools are now present to assess and compare different methodologies in terms of their ecological impact. In this work GAPI index is used. GAPI index [35] has the advantage of covering the whole analytical procedure as compared to the earlier analytical eco-scale.[36]

The proposed MCL method was compared with the official pharmacopoeial methods and six other published methods. Table 7 shows the GAPI index for the proposed and previously published methods. The proposed method has similar greenness comparing to the pharmacopoeial methods and four other reported methods.[1,2,4,6,10,11] The red zones in GAPI pentograms for sampling denote mandatory offline sampling. The advantage of the proposed method is that only 7.5% (v/v) isopropanol is used in the mobile phase compared with published methods.

Compared to pharmacopoeial methods, the developed MLC method can be used for investigation of moxifloxacin and its impurities in the presence of its degradation products. The developed MLC method also achieved a shorter retention time than the pharmacopoeial methods. The use of the biodegradable anionic surfactant SDS (0.15 M) in the aqueous phase increased the surface polarity of the bound C_{18} stationary phase. This change resulted in faster separation of analytes in shorter analysis time,

Table 7. Assessment of the proposed and reported methods-GAPI pictograms

Study	Applied instruments and chromatographic conditions	GAPI
Proposed method	HPLC-DAD using RP-C18 column. Isocratic elution using 92.5% (v/v) biodegradable aqueous mobile phase containing 0.01 M sodium dihydrogen phosphate, 0.15 M sodium dodecyl sulfate (SDS) and 0.5% triethylamine (v/v) and 7.5% of isopropanol (v/v). Sample preparation: in mobile phase.	
Ph. Eur./USP method for moxifloxacin related substances.[1,2]	HPLC-DAD using RP-C18 column. Isocratic elution using mobile phase containing methanol and aqueous solution containing 0.5 g/l tetrabutylammonium hydrogen sulfate, 1 g/l potassium dihydrogen phosphate i 3.4 g/l phosphoric acid (28:72, v/v). Sample preparation: aqueous solution.	
A Rapid RP-HPLC Stability Indicating Method Development and Validation of Moxifloxacin Hydrochloride Related Substances in Finished Dosage Forms.[4]	HPLC-DAD using RP-C18 column. Isocratic elution using mobile phase containing 0.01M potassium dihydrogen orthophosphate as buffer and methanol in the ratio of 70:30. Sample preparation: buffer and methanol in the ratio of 50:50 (v/v).	
A Validated, Specific Stability-Indicating RP-LC Method for Moxifloxacin and Its Related Substances.[5]	HPLC-DAD using RP-C18 column. Gradient elution using mobile phase gradient prepared from 25 mM aqueous sodium dihydrogen orthophosphate dihydrate containing 0.2% triethylamine with orthophosphoric acid (component A) and methanol (component B). The gradient program (time (min)/% B) was: 0/20, 20/50, 30/70, 35/80, 36/20 with a post run time of 5 min. Sample preparation: degassed 60:40 (v/v) mixture of water and acetonitrile.	
Optimization of separation and determination of moxifloxacin and its related substances by RP-HPLC.[6]	HPLC-DAD using RP-C18 column. Isocratic elution using mobile phase, water (+2% triethylamine): acetonitrile 90:10 (v/v). Sample preparation: 0.1% phosporic acid.	
A simple and sensitive HPLC-fluorescence method for the determination of moxifloxacin in human plasma and its application in a pharmacokinetic study.[9]	HPLC-fluorescence detection using RP-C18 column. Isocratic elution using mobile phase composed of 50 mM potassium dihydrogen phosphate buffer pH 2.4 and 100% acetonitrile (77:23, v/v). Sample preparation: the samples were deproteinized by the addition of 500 μl of freshly prepared 6 % trichloroacetic acid in 20 % acetonitrile.	
Stability indicating HPLC method for the simultaneous determination of moxifloxacin and prednisolone in pharmaceutical formulations.[10]	HPLC-DAD using RP-C8 column. Isocratic elution using mobile phase containing mixture phosphate buffer (18 mM) containing 0.1% (v/v) triethylamine, at pH 2.8 (adjusted with dilute phosphoric acid) and methanol (38:62 v/v) Sample preparation: in mobile phase.	
Simultaneous determination of dexamethasone and moxifloxacin in pharmaceutical formulations using stability indicating HPLC method.[11]	HPLC-DAD using RP-C8 column. Isocratic elution using mobile phase containing mixture of phosphate buffer (20 mM) containing 0.1% (v/v) triethylamine, at pH 2.8 and methanol (38.5:61.5 v/v) Sample preparation: in mobile phase.	

enabled the use of isopropanol instead of toxic organic solvent such as acetonitrile, which is more environmentally friendly, and also decreased the ratio required to improve elution to only 7.5% (v/v).[37]

Other reported methods have 3 and 6 red-colored pentograms.[5,9] Compared to the stability indicating method[5], our method has advantages in terms of greenness and also in terms of analysis time. The aim of this research was the same as our proposed method: to develop a method for quantitative analysis of moxifloxacin and its related substances in the presence of degradation products and process-related impurities. This method is based on a gradient mode, while our proposed method is based on an isocratic elution. This method uses acetonitrile, which is not preferred in terms of environmental impact and health safety, while our method uses isopropanol, an environmentally friendly solvent. The analysis time was longer (30 minutes) compared to our method (20 minutes). Compared the proposed method with the method for determination of moxifloxacin in human plasma,[9] the main difference is in the extraction step and sample preparation. Since a very important advantage of MLC concerns sample treatment, without the need for sample pretreatment other than filtration, the proposed MCL method can be considered for use in biological fluids. [3, 12, 38, 39]

4. Conclusions

A new, accurate and selective isocratic eco-friendly MCL method was developed for the determination of moxifloxacin and its related substances in moxifloxacin drug substance in presence of its degradation products. As a result of the central composite design adaptability, a significant acceptability score was achieved, while still obtaining acceptable resolution factors for all critical peaks. Run time was significantly decrease after optimization of the experimental conditions. GAPI was used to compare the proposed method's eco-friendliness to that or other previously reported HPLC methods. The proposed MCL method has advantages in term of greenness since it uses mixture of biodegradable aqueous mobile phase containing SDS, as one of the most researched and best understood widely used anionic surfactant with low price, and low percentage (7.5%) of the more eco-friendly organic solvent, isopropanol. Analysis time was considered as acceptable because the developed method was capable to separate complex mixture containing 11 components. Considering these facts, developed method has lower cost, lower toxicity, greater stability, less negative impact on the environment and greater safety for laboratory use. The developed method also considers the applicability in industrial facilities where selection criteria are based mainly on profit through cost and time. The mathod was found to be simple, selective, precise, accurate and robust. Therefore, this method can be used for routine testing of moxiflox-

acin drug substance. All statistical results were within the acceptance criteria.

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Povzetek

Razvita je bila selektivna in okolju prijazna micelarna HPLC metoda za študije moksifloksacina in sorodnih spojin v prisotnosti njegovih razgradnih produktov. Za optimizacijo eksperimentalnih pogojev je bil uporabljen centralni kompozitni dizajn. Predlagana metoda temelji na izokratni eluciji spojin na C18 koloni z uporabo 92,5 % (v/v) biorazgradljive vodne mobilne faze, ki vsebuje 0,01 M natrijevega dihidrogenfosfata, 0,15 M natrijevega dodecil sulfata (SDS) in 0,5 % trietilamina (v/v) pH 3,5 in 7,5 % izopropanola (v/v) kot okolju prijaznega organskega topila. Hitrost pretoka in volumen injiciranja sta bila 0,6 ml/min ter 5 μl. Poskusi so bili izvedeni pri temperaturi 60 °C, detekcija spojin pa se je vršila pri 295 nm. Optimizirana metoda je bila validirana. Ugotovljeno je bilo, da je metoda primerna za kvantifikacijo moksifloksacina in njemu sorodnih spojin v zdravilni učinkovini moksifloksacin. Indeks zelenih analitičnih postopkov (GAPI) dokazuje superiornost razvite metode v primerjavi z drugimi znanimi metodami.