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ORIGINAL RESEARCH PAPER

Design of experiments and Derringer's desirability function in optimisation and validation of RP-HPLC method for the analysis of enrofloxacin and its impurities

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ABSTRACT

Using the Design of Experiments methodology (*Response-Surface Methodology* and *Derringer's Desirability Function*), a simple, fast and robust RP-HPLC method was developed for the analysis of enrofloxacin (EFC), its impurity A (fluoroquinolonic acid, FQ) and impurity B (ciprofloxacin, CPX). Gradient elution of samples was performed on a Zorbax Eclipse XDB C18 column (150 × 4.6 mm, 3.5 µm) with a mobile phase consisting of 32 mM phosphate buffer pH 3.5 – methanol (0 min-19.6% methanol; 15.5 min-19.6% methanol; 29.5 min-80% methanol; 30 min-19.6% methanol; 35 min-19.6% methanol), delivered at a flow rate of 1.5 mL min^{-1}, wavelength of detection 278 nm (for EFX and CFX) and 265 nm for FQ. A good linear response was achieved in the range 15–35 µg mL^{-1} (EFX) and LOQ-150% for impurities (CFX and FQ). Other validation parameters were also tested: precision, accuracy, sensitivity and robustness. The developed method was shown to be simple, practical and suitable for the analysis of EFC and its impurities (CPX, FQ) in veterinary drugs.

KEYWORDS

enrofloxacin, RP-HPLC, design of experiments, Derringer's desirability function, response surface methodology, optimisation method

1. INTRODUCTION

Enrofloxacin, 1-Cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (EFX), is a synthetic second-generation fluoroquinolone antibiotic, currently approved for use in veterinary medicine. It has a wide spectrum of activity against most Gram-negative bacteria, some Gram-positive bacteria and also Mycoplasma, Chlamydia and Mycobacteria [1]. Bactericidal activity of fluoroquinolones is a result of the inhibition of two intracellular targets: DNA gyrase and DNA topoisomerase IV and a subsequent inhibition of DNA and RNA synthesis [2].

Because of its carboxylic acid moiety (pKa = 6.06) and basic piperazine nitrogen (pKa = 7.70), enrofloxacin is an amphoteric molecule with isoelectric point at pH = 6.85. Presence of piperazine at position C7 of the fluoroquinolone ring improves antimicrobial activity, especially against Pseudomonas. Ethyl group attached to the piperazine nitrogen atom increases lipophilicity and tissue penetration, while reducing central nervous system

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toxicity by reducing binding of the drug to the GABA receptors in brain. Both fluorine and cyclopropyl enhance antibacterial activity [3]. N-Dealkylation yields in an active metabolite ciprofloxacin which is widely used in human medicine.

Enrofloxacin is available on the market in form of tablets and stable parenteral solutions. It is official in the Ph.Eur.10.0 monograph [4] and described as a pale yellow crystal powder, practically insoluble in water, freely soluble in methylene chloride and slightly soluble in methanol. The Pharmacopoeia prescribes tests for specific impurities (A, B, C) and unspecified impurities (E, F, G). Potentiometric method is suggested for determination of enrofloxacin, thin layer chromatography (TLC) for determination of fluoroquinolonic acid (impurity A, FQ) and the reversed-phase high performance liquid chromatographic (RP-HPLC) method for determination of ciprofloxacin (impurity B, CFX) and unspecified impurities (C18 column 150 × 4.6 mm, 5 µm particle size, mobile phase: methanol and phosphate buffer). Due to the use of three different methods, these tests characterize as complex and time consuming. Various methods for determination of enrofloxacin and its impurities in biological samples and pharmaceutical preparations have been described in the literature, with the most recent method including HPLC, LC-MS, spectrophotometric, IR spectroscopic and fluorescence polarization immunoassay methods [5–11].

Design of Experiments (DoE) is a frequently used approach for optimization of analytical methods. The reason for its popularity and wide application came from the possibility to define the behaviour of a system on the basis of a relatively small number of the well-planned experiments [12, 13]. This approach allows monitoring the influence of several factors on the investigated response at the same time and their statistical evaluation. For this purpose, central compositional design (CCD), Box-Behnken design (BBD), Doehlert design, and the other approaches are most frequently used. The Box–Behnken design, unlike the other ones (e.g., CCD) does not contain combinations where all factors are in their high or low levels, which is very useful in avoiding extreme conditions for the experiments that can lead to unsatisfactory results [14, 16]. BBD is often used in combination with the *Response Surface Methodology* (RSM) which represents a set of mathematical and statistical methods used to analyse systems in which a large number of factors influence the response of the system and an aim to optimize that response [14–18].

One of the goals of chromatographic analysis is to achieve good separation of all investigated compounds within the shortest possible time. If the optimal values of the investigated factors are located in different parts of the experimental region and do not overlap, it is difficult to find conditions that simultaneously satisfy several chromatographic goals/responses of the system. In such cases, the multicriteria methodology or the multiple response methodology is used, with an aim of making an appropriate compromise between the given chromatographic objectives [15, 19]. For optimization of chromatographic methods, the most important and most frequently used multi-criteria decision-making methodology is the *Derringer's Desirability Function* (DDF). DDF is applicable to linear and non-linear mathematical models and it does not require selection of a priority system response [19].

After optimization of chromatographic parameters by multicriteria methodology, in which an extensive analysis of the influence of all critical factors (individually and jointly) on the monitored responses of the system was performed, the *one-factor-at-a-time* approach can be applied in testing robustness of the method.

The aim of this study was to investigate chromatographic behaviour of enrofloxacin and its impurities (A and B), in order to define optimum chromatographic conditions suitable for their determination in preparations for use in veterinary medicine. For this purpose, the Design of Experiments methodology was applied for the development of a new RP-HPLC method for the determination of enrofloxacin and its impurities A and B. To the authors' knowledge, this is the first time that a multicriteria approach was used in the optimization of the method for testing enrofloxacin and its impurities A and B.

2. EXPERIMENTAL

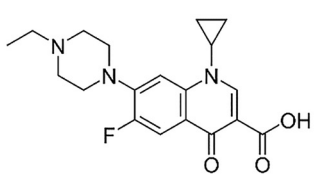
2.1. Chemicals and reagents

The analysed substances were: Enrofloxacin (purity 98.5%), Dr. Ehrenstorfer GmbH (stock number: 13170000; batch number: 20606), Germany, and impurities: 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (fluoroquinolone acid (impurity A), purity 99.6%) CRS European Pharmacopoeia reference standard (stock number: MM0018.1; batch number: 18.01.08.05), LCG GmbH, Germany; ciprofloxacin USP reference standard (impurity B), purity 99.8% (stock number: 1134313; batch number: HOE306), USA. Chemical structures of Enrofloxacin and its impurities A and B are shown in Fig. 1.

The mobile phase and the solvents were prepared from methanol, suitable for HPLC, purity ≥99.9%, (J.T. Baker, Netherlands). Triethanolamine purity ≥99.0%, (Fisher Chemical, USA), sodium hydroxide (Merck, Germany), ortho-phosphoric acid, 85% (Merck, Germany), and HPLC grade water. Enrocin-S 10% solution for injection® (Veterinary Institute Subotica, Serbia) were used for analysis. Placebo components: sodium hydroxide, diethanolamine, benzyl alcohol, propylen glycol, water for injections (Veterinary Institute Subotica, Serbia). All the reagents utilized in this study were of analytical grade.

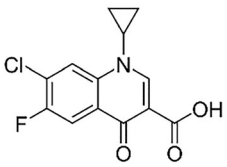
2.2. Chromatographic conditions

The experiments were performed on the chromatographic system Agilent 1200 series consisting of the HPLC Pump, the Quaternary Pump, the ALS Autosampler, and the UV/VIS DAD detector. The Agilent ChemStation software was used for data collection. The analytical column was Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, 3.5 µm particle size) Agilent Technologies, USA. Throughout



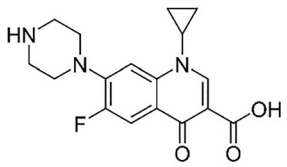
Enrofloxacin

1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid



Fluoroquinolonic acid (impurity A)

7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid



Ciprofloxacin (impurity B)

1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid

Fig. 1. Chemical structures of enrofloxacin, fluoroquinolonic acid (impurity A) and ciprofloxacin (impurity B)

the complete experimental procedure, the following instrumental conditions were maintained: the flow rate of mobile phase 1.5 mL min^{-1}, the injection volume 20 µL, the column temperature 40 °C and the UV detection at 265 nm (enrofloxacin, ciprofloxacin) and 278 nm (fluoroquinolone acid). The experimental plan was developed according to BBD (for optimization) and the *one-factor-at-time* approach (for robustness).

2.3. Mobile phase

Mobile phase consisted of two solvents in the gradient approach mode: solvent A - water phase (2.2 mL ortho-phosphoric acid added in 900 mL HPLC grade water, and pH value set to 3.5 with triethanolamine; then made up to 1,000 mL) and solvent B - methanol. The prepared solvents were filtered through a 0.45 µm nylon membrane filter (Whatman, England) and degassed for 15 min on an ultrasonic bath.

2.4. Standard solutions

Stock solutions. Stock solutions of EFX (1 mg mL^{-1}), FQ (0.04 mg mL^{-1}) and CFX (0.05 mg mL^{-1}) were prepared by dissolving each standard in the 0.1 M NaOH and dilution with methanol-water (20:80, V/V). Solutions were protected from light and stored at 2–8 °C.

Working solutions. Robustness of the method was tested by preparing the pH buffer solutions: pH 3.3, 3.5 and 3.7 (2.2 mL ortho-phosphoric acid added in 900 mL HPLC grade water, and the pH value set to 3.3, 3.5 and 3.7 with triethanolamine; then made up to 1,000 mL).

For estimation of linearity, five solutions containing EFX (15; 20; 25; 30; 35 µg mL^{-1}), FQ (0.25; 1.3; 2.0; 2.5; 3.0; 3.8 µg mL^{-1}) and CFX (0.5; 2.0; 3.5; 5.0; 6.0; 7.5 µg mL^{-1}) were prepared in the mobile phase (methanol-water 20:80, V/V).

Estimation of accuracy was performed using three series of three solutions containing placebo, EFX (20 µg mL^{-1}, 25 µg mL^{-1} and 30 µg mL^{-1}), FQ (0.25 µg mL^{-1}, 2.5 µg mL^{-1} and 3.0 µg mL^{-1}) and CFX (0.5 µg mL^{-1}, 5.0 µg mL^{-1} and 6.0 µg mL^{-1}). All solutions were prepared in the mobile phase (methanol-water 20:80, V/V).

For estimation of precision two types of solutions were used:

a) six solutions of EFX (25 µg mL^{-1}) were prepared in the mobile phase (methanol-water 20:80, V/V) and spiked with FQ (2.5 µg mL^{-1}) and CFX (5 µg mL^{-1});
b) six solutions of EFX (25 µg mL^{-1}) were prepared in the mobile phase (methanol-water 20:80, V/V) and spiked with FQ (0.25 µg mL^{-1}) and CFX (0.5 µg mL^{-1}).

Solutions for estimation of the limit of detection (LOD) and the limit of quantification (LOQ) were prepared separately for each compound in the concentrations 1.0; 0.5; 0.25; 0.10; 0.05; 0.025; 0.01 µg mL^{-1}. All solutions were prepared in the mobile phase (methanol-water 20:80, V/V).

Placebo mixture (sodium hydroxide, diethanolamine, benzyl alcohol, propylene glycol, water for injections) for estimation of selectivity was prepared in the concentration ratio corresponding to the content in the pharmaceutical dosage form (Enrocin-S 10% solution for injection®). Standard solutions of EFX (25.0 µg mL^{-1}), FQ (2.5 µg mL^{-1}) and CFX (5 µg mL^{-1}) were used to prove the selectivity.

Real sample Enrocin-S 10% solution for the injection® was used for qualitative and quantitative analysis of EFX and its quality control. Three identical solutions were prepared in the mobile phase (methanol-water 20:80, V/V) in the concentration of 25.0 µg mL^{-1}.

2.5. Software

Experimental design and data analysis were performed in the Design-Expert 7.0.0 software (Stat-Ease Inc., Minneapolis, MN, USA). The MarvinSketch 15.4.13. (ChemAxon Kft, Budapest, Hungary) software was used for estimation of the log*P*, log*D* and p*Ka* values.

3. RESULTS AND DISCUSSION

3.1. Preliminary experiments

Development and optimization of the HPLC method requires determination of optimal chromatographic conditions enabling good separation of the tested compounds in

an acceptable time of analysis. This task is very demanding, due to similar properties (e.g., similar p*Ka* and log*P* values) and similar chromatographic behaviour of analytes. Separation in RP-HPLC is based on differences in lipophilicity values among the test compounds. Based on their log*P* value, the elution order of the compounds can be assumed, which means that the compounds with the highest log*P* value (i.e., the most lipophilic ones), are eluted as the last ones in the RP-HPLC system. By comparing the log*P* values for EFX (log*P* = 2.04), CFX (log*P* = 1.32) and FQ (log*P* = 2.22), it can be concluded that the order of their elution in the RP-HPLC system should be CFX → EFX → FQ.

Preliminary experiments were conducted on several C8 and C18 RP-HPLC columns and the best results were achieved with the Zorbax Eclipse XDB-C18 (150 nm × 4.6 mm, 3.5 µm particle size) column. The UV spectra of all tested compounds were recorded in the wavelength range 190–400 nm. Two wavelengths of the absorption maxima were selected: 278 nm (for EFX and CFX) and 265 nm (for FQ).

Acetonitrile was tested as an organic component of mobile phase to obtain sharp and symmetrical peaks of the investigated compounds, but also to shorten the analysis time. However, it was methanol only which enabled satisfactory separation of EFX from its impurities (FQ, CFX) and provided good as values. Although with methanol the analysis time is slightly longer, its use is more economical. In order to reduce tailing factor of EFX and its impurities, triethanolamine was used, which reduces intermolecular interactions between analytes and the silanol groups on the stationary phase surface. Initially, isocratic elution was used, but due to high retention time of FQ, gradient elution was applied. It was concluded that after elution of CFX and EFC from the column, increasing methanol content in mobile phase significantly accelerates elution of FQ and shortens an overall analysis time.

In the next step, the influence of ionic strength and the pH value of the aqueous buffer solution was examined on the retention behaviour of the tested compounds. Ionic strength was tested in the concentration range 15–30 mM, and the pH value in the range 2.5–7.5 (the pH value of the tested solutions was adjusted with concentrated acetic acid). It was concluded that both factors have a significant influence on retention parameters. By adjusting the pH value of the buffer, ionization of the analyte can be favoured, resulting in sharper and more symmetrical peaks. At the pH range 2.5–3.5, EFX and CFX are mostly ionized, while FQ is in a non-ionized form. Other parameters of the chromatographic system were also tested: column temperature in the range 30–40 °C and mobile phase flow rate in the range 1.0–1.5 mL min^{-1}. The injection volume was kept constant at 20 µL.

The results obtained in the course of preliminary research allowed to select a set of critical responses of the chromatographic system (*CRs*):

1. $Rs_{CFX,EFC}$ – the resolution between CFX and EFC,
2. tR_{FQ} – the retention time of FQ (the analysis time).

Critical method parameters, i.e. investigated factors (*IFs*) of the chromatographic system which had to be monitored during method optimization were also identified:

1. pH value of water phase,
2. triethylamine concentration in water phase (buffer concentration),
3. column temperature,
4. methanol ratio in mobile phase.

All other factors were kept constant: mobile phase flow rate (1.5 mL min^{-1}), detection wavelengths (278 nm, 265 nm) and the injection volume (20 µL).

3.2. Method optimization

In order to examine the influence of *IFs* on *CRs*, the *Design of Experiments* (DoE) method was used by employing BBD. Following the preliminary studies, value ranges for each *IFs* were defined:

2.5–3.5 for pH of water phase,
15–20 mM for triethylamine concentration in water phase (buffer concentration),
18–22% methanol content in mobile phase,
30–40 °C column temperature.

From the obtained results (Table 1), it comes out that the system is sensitive to the changes in all tested factors (the $Rs_{CFX,EFC}$ value varies in the range of 1.8–6.9), and the tR_{FQ} value in the range of 27.5–38.0 min. To investigate the influence of each individual *IFs* and its interactions with *CRs*, the multiple linear regression and the least squares methods were applied, using the parameters that describe chromatographic behaviour of EFX and its impurities.

The resulting models were validated using the ANOVA test (Table 2). The obtained high values of determination coefficients R^2, the *adjusted* R^2 and the *predicted* R^2 indicate good correlation of the experimentally determined and the model-calculated response values. Factors and factor interactions that were shown as significant for each examined response ($P \leq 0.05$), were identified and used to define the equations of the quadratic response model:

$$Rs_{CFX,EFC} = 3.90 + 1.51A - 0.82C + 0.63D - 0.40CD + 0.50A^2 - 0.37D^2 \quad (1)$$

$$tR_{FQ} = 32.5 + 0.75\mathrm{A} - 1.92\mathrm{B} - 4.00\mathrm{C} + 0.88\mathrm{C}^2 \quad (2)$$

A, pH value of water phase; B, temperature of column; C, methanol content in mobile phase, and D, buffer concentration.

The quadratic terms of factors A^2, C^2, D^2 (Eqs (1) and (2)) indicate the presence of a nonlinear relationship between A, C and D, and the analysed system responses. Factors with the (+) sign indicate that this change has a concave shape (extreme value is the function minimum), while factors with the (−) sign indicate that this change has a convex shape (extreme value is the function maximum).

From Eq. (1), it can be seen that factors A, C, and D, and factors interaction CD have a significant influence on the

Table 1. Plan of the Box–Behnken design experiments and the obtained data

No.	Factors				Responses	
	A: pH value	B: Temperature (°C)	C: methanol content (%)	D: buffer concentration (mM)	$Rs_{CFX,EFX}$	tR_{FQ}
1	3.00	35.00	22.00	35.00	3.1	29.0
2	2.50	30.00	20.00	25.00	2.8	33.5
3	2.50	35.00	20.00	15.00	1.8	31.5
4	3.00	30.00	20.00	15.00	3.0	34.0
5	3.00	35.00	20.00	25.00	4.0	32.5
6	3.00	30.00	20.00	35.00	4.1	34.5
7	3.00	30.00	18.00	25.00	4.8	40.0
8	3.50	35.00	18.00	25.00	6.9	38.0
9	3.50	35.00	20.00	15.00	4.6	33.5
10	3.00	40.00	22.00	25.00	2.8	27.5
11	2.50	35.00	22.00	25.00	2.0	30.0
12	3.00	40.00	18.00	25.00	4.6	35.0
13	3.50	35.00	22.00	25.00	4.7	31.0
14	2.50	35.00	18.00	25.00	3.6	38.0
15	3.00	35.00	18.00	35.00	5.3	37.5
16	3.00	30.00	22.00	25.00	2.8	30.0
17	3.00	35.00	18.00	15.00	3.0	35.5
18	3.00	40.00	20.00	35.00	3.2	30.5
19	3.50	35.00	20.00	35.00	6.6	33.5
20	3.00	35.00	20.00	25.00	4.0	32.5
21	3.00	35.00	20.00	25.00	3.7	32.5
22	3.00	40.00	20.00	15.00	3.2	30.0
23	3.50	40.00	20.00	25.00	6.0	31.5
24	3.50	30.00	20.00	25.00	5.5	35.5
25	3.00	35.00	22.00	15.00	2.4	28.5
26	2.50	40.00	20.00	25.00	2.7	30.0
27	2.50	35.00	20.00	35.00	3.3	31.0

$Rs_{CFX,EFX}$ – resolution between CFX and EFC; tR_{FQ} – the retention time of FQ.

resolution factor between CFX and EFC. By increasing the pH value of water phase and decreasing the methanol content in mobile phase, resolution between CFX and EFC increases. An increase of buffer concentration will lead to the increase in the $Rs_{CFX,EFC}$ value up to the point when the nonlinear effect of buffer concentration should be considered. With the increase of both factors, C and D, the $Rs_{CFX,EFC}$ value decreases.

The retention time of FQ (Eq. (2)) is influenced by factors A, B and C. By increasing the pH value of the aqueous buffer solution and by decreasing column temperature, the retention time increases, while an increase in the methanol content will lead to the decrease in retention time up to the point when the tR_{FQ} value starts to increase, because of the nonlinear contribution of this factor.

Based on the obtained results of the ANOVA test, the 3D-graphs that represent the relationship between *IFs* and *CRs* were constructed using *Response Surface Methodology* (Fig. 2).

From the 3D-graphs obtained for each observed response, it can be concluded that there is a significant two-factor interaction between factors C and D (methanol content in mobile phase and triethylamine concentration in the water phase/buffer concentration).

The pH value of the aqueous buffer solution has the highest influence on the response $Rs_{CFX,EFC}$ value (an increase of the pH value results in an increase of resolution of critical pair). Other factors and factor interactions do not significantly affect the response, with an exception of factor interaction CD. From Fig. 2A3, it can be seen that by the combined action of these two factors (reducing methanol content in mobile phase and increasing concentration of the buffer), resolution of the critical pair ($Rs_{CFX,EFX}$ = 5.4) significantly grows, while this increase is not expressed by their individual action, nor in combination with the other factors (Fig. 2A1 and 2A2).

When analysing the retention time of FQ (tR_{FQ}) and the 3D-graph, it can be concluded that with an increase of the methanol content in mobile phase and the decrease in the pH value of the aqueous buffer solution, the analysis time becomes shorter. The ionic strength of the buffer is not such a significant factor and from Fig. 2B2 and 2B3, it can be seen that by increasing buffer concentration, the analysis time is slightly prolonged (by less than 1 min).

3.3. Multicriteria methodology–Derringer's Desirability function

In order to obtain global optimum of chromatographic conditions (i.e., the space in which all goals set for the monitored responses of the system are met), DDF was applied. The obtained results are presented in Table 3 and Fig. 3.

Table 2. Coefficients of the obtained quadratic models in terms of the coded factor values and statistical analysis

	$Rs_{CFX,EFX}$		tR_{FQ}	
	coeff.	*P*-value	coeff.	*P*-value
b_0	3.90	<0.0010	32.5	<0.0010
A	1.51	<0.0010*	0.75	0.0022*
B	−0.04	0.6572	−1.92	<0.0010*
C	−0.82	<0.0010*	−4.00	<0.0010*
D	0.63	<0.0010*	0.25	0.2217
AB	0.15	0.3629	−0.13	0.7163
AC	−0.15	0.3629	0.25	0.4711
AD	0.13	0.4459	0.13	0.7163
BC	0.05	0.7580	0.63	0.0875
BD	−0.27	0.1085	0.00	1.0000
CD	−0.40	0.0268*	−0.38	0.2861
A^2	0.50	0.0036*	0.50	0.1113
B^2	−0.13	0.3656	−0.13	0.6751
C^2	−0.07	0.6362	0.88	0.0109*
D^2	−0.37	0.0204*	−0.50	0.1113
R^2	0.9742		0.9792	
$R^2_{adj.}$	0.9440		0.9550	
$R^2_{pred.}$	0.8557		0.8805	
SD	0.32		0.67	

$Rs_{CFX,EFX}$ – resolution factor between CFX and EFX; tR_{FQ} – retention time of FQ; R^2 – coefficient of determination; $R^2_{adj.}$ – adjusted R^2 which represent R^2 values adapted to the total number of experiments; $R^2_{pred.}$ – predicted R^2 indicates how well a regression model predicts responses for new observations; SD – standard deviation; b_0 – section; A – linear term of factor A (pH value of water phase); B – linear term of factor B (temperature of column); C – linear term of factor C (methanol content in the mobile phase); D – linear term of factor D (triethylamine concentration in the water phase/buffer concentration); AB – interaction member of factors A and B; AC – interaction member of factors A and C; BC – interaction member of factors B and C; A^2 – quadratic member of factor A; B^2 – quadratic member of factor B; C^2 – quadratic member of factor C; D^2 – quadratic member of factor D.
* significant coefficient for *P*–value ≤ 0.05

Table 3 shows acceptable ranges for investigated factors and the achievement goals for selected responses. The shape of the desired response function for each observed system response is defined by the selected values of the weighting coefficients. Values of the significance coefficients which are used to calculate global optimum, are also determined. Combination of factors that give global optimum (D = 1,000) was defined, as follows: methanol content, 19.70%; pH of water phase, 3.39; column temperature, 39.98 °C; buffer concentration, 32.16 mM (Fig. 3). For these conditions, the predicted response values were: $Rs_{CFX,EFX}$ = 6.29 and tR_{FQ} = 27.50 min.

After the analysis of all the results obtained, optimal chromatographic parameters were finally established:

column:	Zorbax Eclipse XDB C18 (150 mm × 4.6 mm, 3.5 µm)
mobile phase:	32 mM phosphate buffer, pH 3.5 – methanol (two separated channels)
gradient:	0 min-19.6% methanol; 15.5 min-19.6% methanol; 29.5 min-80% methanol; 30 min-19.6% methanol; 35 min-19.6% methanol
flow rate:	1.5 mL min^{-1}
column temperature:	40°C
injection volume:	20 µL
wavelength:	278 nm for EFX and CPX; 265 nm for FQ.

The experimental chromatogram obtained under the selected optimal working conditions is shown in Fig. 4.

After defining the optimal chromatographic conditions, robustness of the method was assessed, using the *one-factor-at-time* approach. The influence of each factor was examined separately, while the other factors were kept at constant levels. Three chromatographic parameters were selected for this purpose: column temperature 40 ± 4 °C, mobile phase flow rate 1.5 ± 0.1 mL min^{-1} and pH value of the aqueous buffer solution 3.5 ± 0.2. Changes in the retention time (tR) of all tested compounds were observed, as well as changes in resolution of the critical pair ($Rs_{CFX,EFC}$). The obtained values were compared with those coming from the optimal chromatographic conditions. All the obtained results are given in Table 4.

From the assessment results referring to robustness (Table 4), it can be concluded that all the values of the tracked responses are satisfactory and acceptable, which confirms that the method is robust.

Selectivity of the method was confirmed by comparing the chromatogram of the investigated compounds with the chromatogram of placebo (Fig. 5). It can be seen that there is no overlapping of chromatographic peaks between tested compounds and placebo at the retention times corresponding to EFX, CFX and FQ. Thus it can be concluded that the method is selective.

Testing of linearity, accuracy and precision, and determination of the limit of detection (LOD) and the limit of

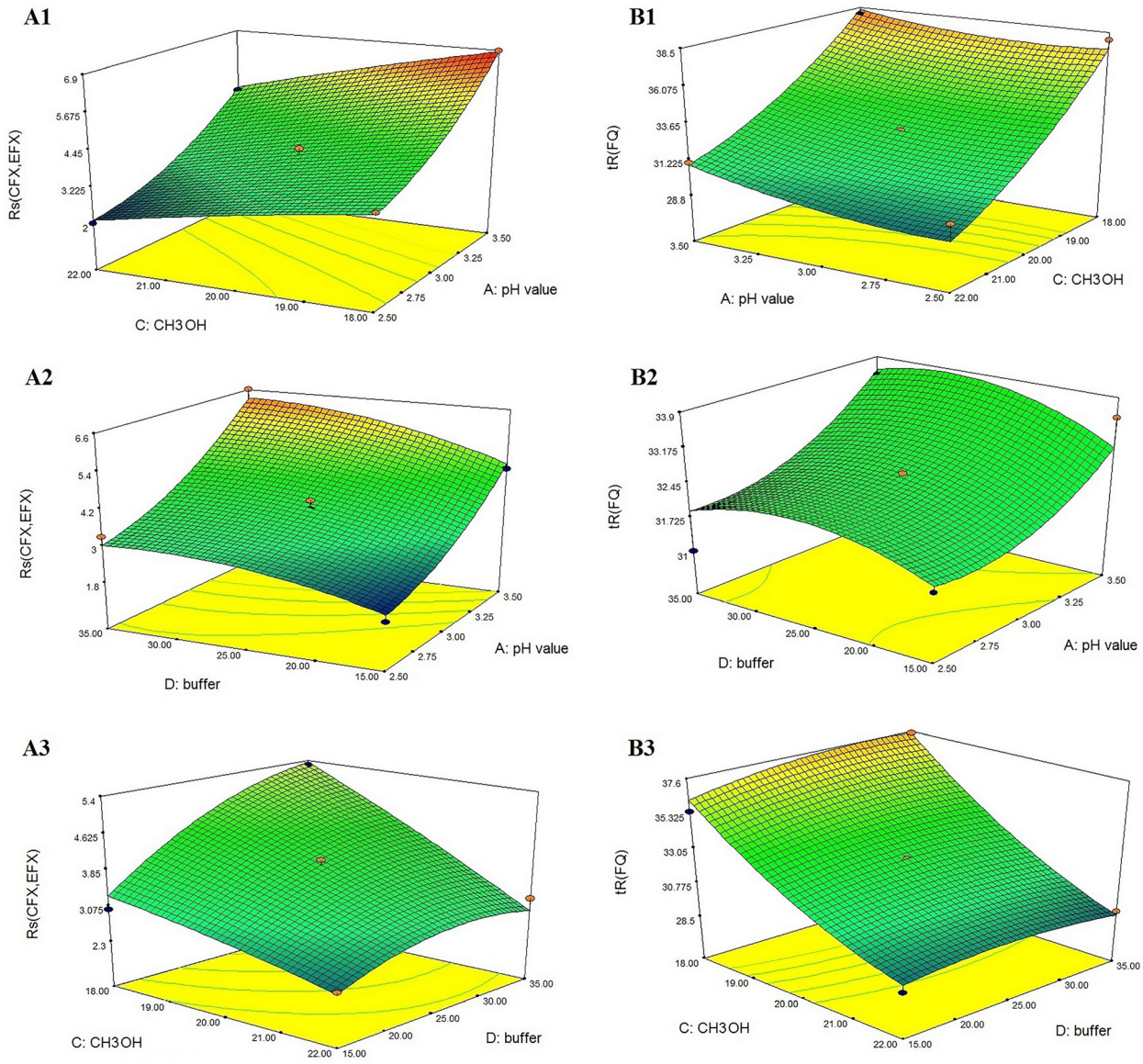


Fig. 2. The 3-D graphs for critical responses in function of two critical parameters of the chromatographic system (content of methanol in the mobile phase [%], concentration of buffer in water phase [mmol L^{-1}] and pH-value of water phase); A1–A3: $Rs_{CFX,EFX}$ – resolution factor between CFX and EFX; B1–B3: tR_{FQ} – retention time of FQ

Table 3. Derringer's Desirability Function – the limit values and goals for determination of the global optimum

	Variables	Range		Weight	Goal	Relative importance
		Lower limit	Upper limit			
Factors (inputs)	pH value	2.5	3.5	1	in range	3
	Temperature	30	40	1	in range	3
	Methanole (%)	18	22	1	in range	3
	Buffer concentration (mM)	15	35	1	in range	3
Responses (outputs)	$Rs_{CFX,EFX}$	1.8	6.9	1	> 1.2	4
	tR_{FQ}	26.5	40.0	1	min.	5

$Rs_{CFX,EFX}$ – resolution between CFX and EFC; tR_{FQ} – the retention time of FQ.

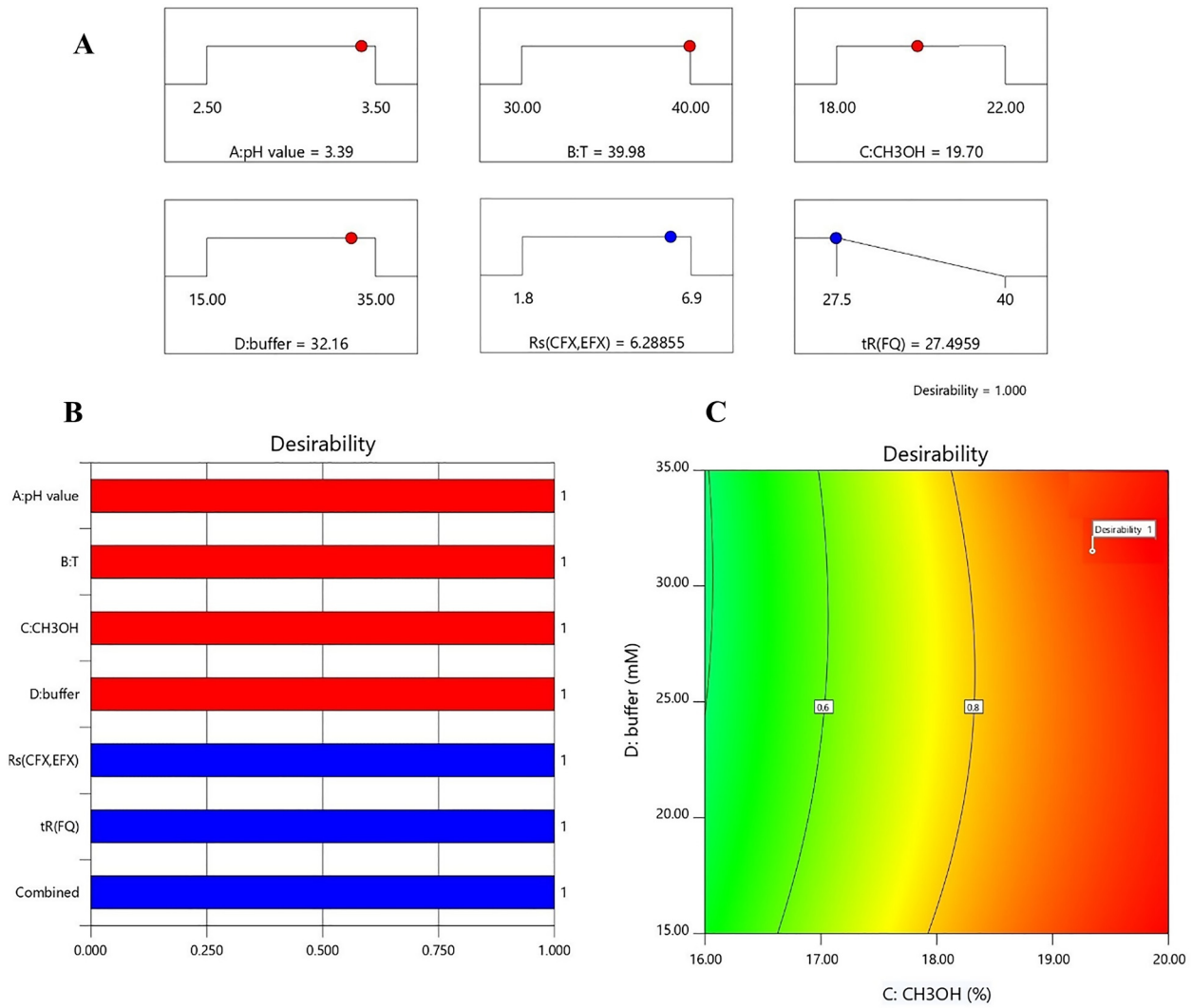


Fig. 3. A) Graphical presentation of the optimal mobile phase composition, as well as predicted responses and the corresponding adopted constraints; B) Desirability chart - showing that all requirements were met and the global optimum was achieved; C) The 2D-graph of desirability space in the function of two critical parameters of the chromatographic process (content of methanol in the mobile phase [%] and concentration of buffer [mmol L^{-1}])

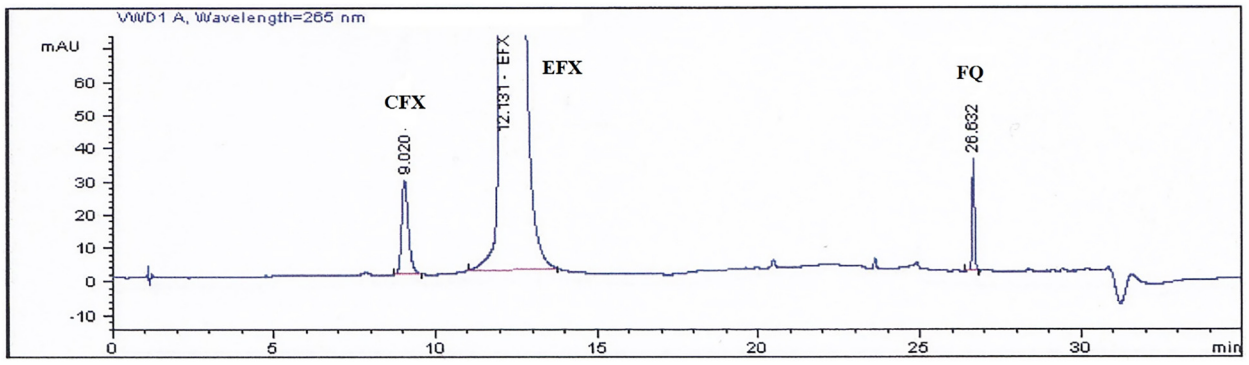


Fig. 4. The experimentally obtained chromatogram under the optimal working conditions

Table 4. Results of the robustness test for determination of the enrofloxacin content

Chromatographic conditions	Variables	EFX		CFX	FQ
		$Rs_{CFX,EFX}$	tR_{EFX}	tR_{CFX}	tR_{FQ}
Temperature (°C)	36	5.75	13.65	10.19	26.86
	40	5.95	12.13	9.02	26.63
	44	5.84	10.81	7.88	26.39
Mobile phase flow rate (mL min^{-1})	1.4	6.25	12.98	9.53	26.94
	1.5	5.95	12.13	9.02	26.63
	1.6	6.27	11.35	8.32	26.36
pH value of water phase	3.3	5.97	11.68	8.86	26.62
	3.5	5.95	12.13	9.02	26.63
	3.7	8.07	12.73	8.66	26.62

$Rs_{CFX,EFX}$ – resolution between CFX and EFC; tR_{EFX} – the retention time of EFX; tR_{CFX} – the retention time of CFX; tR_{FQ} – the retention time of FQ.

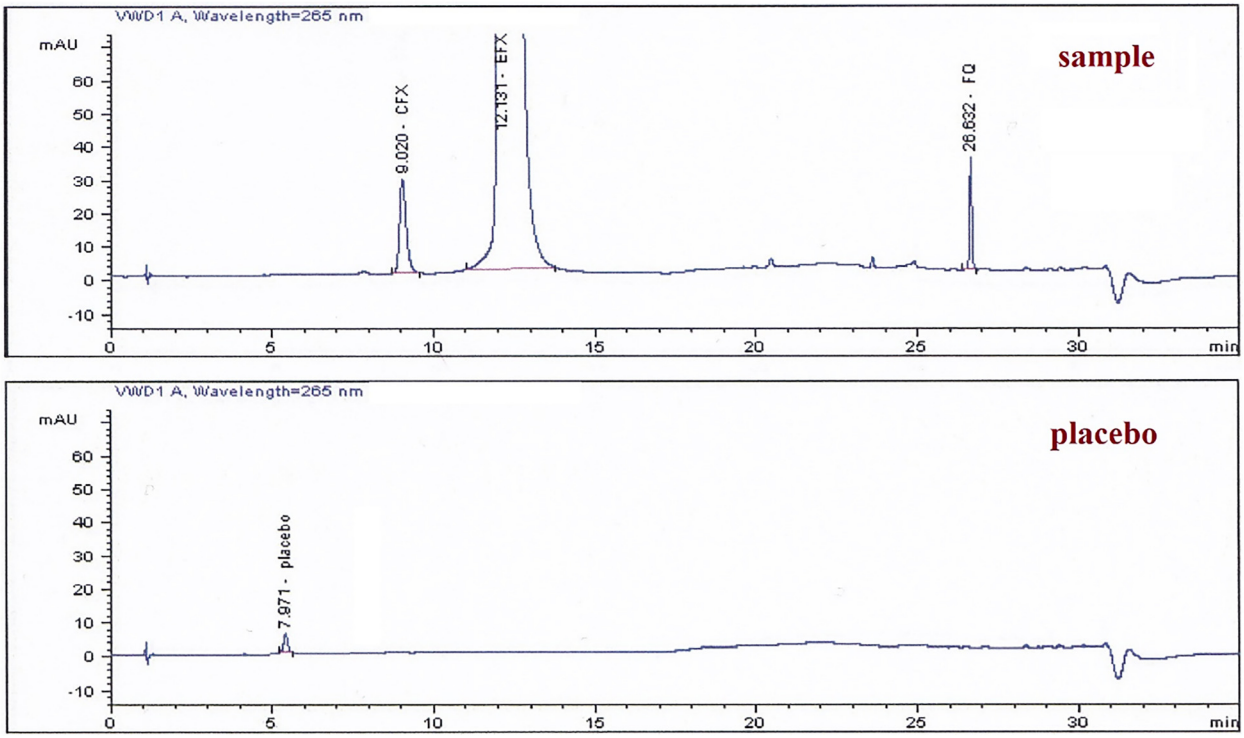


Fig. 5. The chromatogram which confirms selectivity of the RP-HPLC method

quantification (LOQ) for EFX, CFX and FQ was performed, and the results obtained are presented in Table 5.

Determination of the LOD and LOQ values for the investigated impurities was done by combination of experimental work and evaluation of the signal/noise ratio. The reported values obtained for both impurities (CFX and FQ) are very low. It is of great importance to emphasize that both LOQ values are ca. 10 times lower than the values defined by Eur.Ph.10.0 (enrofloxacin monograph). From this result it can be concluded that the method is sensitive enough to be used for quality control of enrofloxacin in the drug products.

Linearity of the method was assessed using different concentration ranges of the test compounds: for EFX, it was 15.0–35.0 μg mL^{-1}, while for CFX and FQ, the linearity was tested in the range from LOQ to 150% of the concentration selected for method development (for CFX, 5.0 μg mL^{-1} and for FQ, 2.5 μg mL^{-1}). Regression analysis of the obtained data (Table 5) shows that for all three investigated compounds, linear dependence between peak areas and concentration (within the defined range) was observed. Linearity coefficients for all the obtained calibration curves are higher than 0.998 and the *P*-value also meets the requirement for linearity ($P > 0.05$) [20].

Accuracy of the method was established by using three different concentration values for each compound: EFX (20 μg mL^{-1}, 25 μg mL^{-1} and 30 μg mL^{-1}), FQ (0.25 μg mL^{-1}, 2.5 μg mL^{-1} and 3.0 μg mL^{-1}), and CFX (0.5 μg mL^{-1}, 5.0 μg mL^{-1} and 6.0 μg mL^{-1}). The obtained recovery values were in the range of 98%–102% for EFX and 90%–110% for CFX and FQ. The relative standard deviation (RSD%)

Table 5. Validation parameters

	Enrofloxacin (EFX)	Impurity A (CFX)		Impurity B (FQ)	
LOD (μg mL^{-1})	–	0.20		0.10	
LOQ (μg mL^{-1})	–	0.50		0.25	
Linearity					
Concentration range (μg mL^{-1})	15.0–35.0	LOQ (0.5)–7.5		LOQ (0.25)–3.8	
y = ax + b	y = 101577x + 25.30	y = 114.7x − 12.38		y = 65.86x + 1.84	
r	0.9991	0.9994		0.9999	
P-value	0.71	0.25		0.25	
Accuracy					
80% or LOQ (μg mL^{-1})					
Recovery (%)	99.10 (80%)	99.8 (LOQ)		107.1 (LOQ)	
RSD (%)[a]	0.89	0.34		2.18	
100% (μg mL^{-1})					
Recovery (%)	98.9	98.0		98.8	
RSD (%)[a]	0.32	1.24		0.84	
120% (μg mL^{-1})					
Recovery (%)	99.3	102.3		99.9	
RSD (%)[a]	0.29	0.87		0.97	
Precision	100%	LOQ	100%	LOQ	100%
Concentration (μg mL^{-1}):					
I day (μg mL^{-1})	24.85	0.499	4.99	0.254	2.54
II day (μg mL^{-1})	24.87	0.499	4.99	0.254	2.54
RSD (%)[b]	0.83	3.79	1.00	2.43	1.63
Enrocin-S® 10% solution for injection	99.13% (99.13 μg mL^{-1})	0.12% unspecified impurities: 0.14%[c] total impurities: 0.44%[c]		<LOD	

EFX - enrofloxacin; CFX - ciprofloxacin; FQ - fluoroquinolone acid; LOD – limit of detection; LOQ – limit of quantification; *r* – correlation coefficient (>0.99 for active ingredients, >0.98 for related compounds [19, 21]); *P*-value ≥ 0.05 meets the requirement for linearity; [a] RSD - relative standard deviation for accuracy (<2% for active ingredients, <15% for related compounds); [b] RSD-relative standard deviation for precision (≤1.0% for active ingredients, <10% for related compounds) [19, 22]; [c] - unspecified impurities <0.20% and total impurities <1.0% [4].

values were also in acceptable ranges (RSD <2% for active ingredients, RSD <15% for the related compounds), in that way confirming the accuracy of the method.

For the precision tests, six samples of EFX (25 μg mL^{-1}), CFX (LOQ and 5.0 μg mL^{-1}) and FQ (LOQ and 2.5 μg mL^{-1}) were prepared. The obtained recovery and RSD% values confirmed method precision (Table 5).

Finally, the developed method was applied for the analysis of the Enrocin-S 10% solution for injection (the qualitative and quantitative analysis was performed). The obtained results (Table 5) show that the method is applicable for the analysis of the Enrocin-S 10% solution for injection, proving its sensitivity, specificity, accuracy and precision.

4. CONCLUSION

Using the Design of Experiments methodology, the *Response-Surface Methodology* and the *Derringer's Desirability Function*, the RP-HPLC method for the simultaneous qualitative and quantitative analysis of enrofloxacin and its impurities A and B was successfully developed and optimized. The Design of Experiments methodology successfully identified critical parameters of the chromatographic system. After optimization of chromatographic parameters, robustness of the method was tested and method was validated. The method proved to be selective, accurate, precise and sensitive, which was confirmed in the routine analysis of the Enrocin-S 10% solution for injection.

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