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Optimization and validation of a CZE method for rufloxacin hydrochloride determination in coated tablets

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Abstract

A simple and rapid capillary electrophoresis method with UV detection was developed and validated for the determination of rufloxacin hydrochloride in coated tablets. An experimental design strategy (Doehlert design and desirability function) allowed the analytical parameters to be simultaneously optimized in order to determine rufloxacin hydrochloride with high peak area/migration time ratio, good efficiency and short analysis time. Optimized analyses were run using boric acid 0.10 M adjusted to pH 8.8 as BGE and setting voltage and temperature at 18 kV and 27 °C, respectively. Pefloxacin mesylate was used as internal standard and run time was about three minutes. The method was validated for the drug substance and the drug product according to the ICH3 guidelines. Robustness was tested by experimental design using an eight-run Plackett–Burman matrix. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Experimental design; Capillary electrophoresis; Rufloxacin hydrochloride; Optimization; Validation

1. Introduction

Rufloxacin, 9-fluoro-2,3-dihydro-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzothiazine-6-carboxylic acid, is a long-acting fluoroquinolone antibacterial. This drug has been shown to possess marked in vivo activity against Gram-negative and Gram-positive bacteria [1,2]. Rufloxacin is given by mouth as the hydrochloride (RU) in the treatment of susceptible infections in an initial dose of 400 mg followed by 200 mg daily, and is marketed in 200 mg coated tablets.

Reported methods for the determination of rufloxacin consist in HPLC [3–12], UV-spectroscopy [13,14], fluorimetry [15]. Some of us have described the adsorptive stripping voltammetric method with a hanging mercury drop electrode [16].

Several reports have been dedicated to quinolones analysis by capillary electrophoresis

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(CE). This technique has been used to determine ciprofloxacin in a pharmaceutical formulation [17] and for the enantioselective separation of ofloxacin and DU-6859, a new generation quinolone, using either vancomycin [18] or the mixture γ-cyclodextrin-Zn(II)-D-phenylalanine [19] as chiral selector. CE made it possible to calculate dissociation constants of quinolones in water and hydro-organic media and to evaluate the effect of pH on the electrophoretic mobility, to predict the optimum pH for the separation of a series of quinolones [20–23]. Other CE methods were developed for the separation and/or the quantitative determination of some quinolone antibiotics [24–32]; however, no attention has yet been paid to quantitative determination of RU.

The aim of this paper was mainly to optimize and validate, using the response surface methodology, the developed rapid capillary zone electrophoresis (CZE) method, suitable for the assay of RU in coated tablets.

Although the use of a highly efficient separating method for the assay of a single active achiral ingredient in tablets is a relatively easy task, it is very doubtful that a univariate optimization of the experimental conditions is, also in this case, the best way to proceed. In fact, even if a univariate strategy can appear the simplest approach, actually, this is not the correct way to avoid pitfalls during an experimental work [33,34]. With a univariate strategy, the 'optimum conditions' are obtained from a number of experiments depending on the experience and luck of the researcher. For these reasons, a multivariate strategy, such us an experimental design, represents the best way to approach the optimization of the variables. In addition, experimental design is a very simple strategy, the use of which should be encouraged through the demonstration that it is not a complicated statistical approach, but, with an appropriate software, it is a simple tool in the hands of the researcher. In particular, in this work experimental design was used to optimize the different electrophoretic parameters for the CZE analysis of RU with the aim of improving the method efficiency, peak area/migration time ratio and speed.

2. Experimental

2.1. Chemicals and solutions

All chemicals used were of analytical-reagent grade with no further purification.

Working standards of RU, coated tablet excipients (microcrystalline cellulose, maize starch, lactose monohydrate, carboxymethylcellulose sodium, maize starch pre-gelatinized, magnesium stearate, hydroxypropyl methylcellulose, titanium dioxide and polyethylene glycol 400) and pharmaceutical dosage form (QARI®) containing 200 mg of RU for each coated tablet were obtained from Mediolanum Farmaceutici (Milan, Italy). Pefloxacin mesylate (PE) was obtained from Prodotti Formenti (Milan, Italy) and used as internal standard.

Reagent-grade water was obtained with a Milli-Q system (Millipore/Waters, Milford, MA, USA) and was used to prepare all solutions.

Since buffer pH was considered to be very important, pH 8.3–8.9, 0.04–0.10 M BGE examined during the optimization step were prepared adding an accurately weighed amount of boric acid to a 100 ml volumetric flask to obtain the desired final concentration; pH was adjusted with 1 M NaOH before filling up to the volume with water.

The running buffer consisted of 0.10 M boric acid adjusted to pH 8.8 with 1 M NaOH. Standard stock solution of RU and PE were prepared in water at concentration of 0.10 and 0.11 g l^{-1}, respectively. These solutions were stored at 4 °C and used within 3 days. A working standard solution was prepared daily by diluting standard stock solutions with water in order to obtain the desired final concentrations.

BGE and working standard solution were filtered through 0.45 μm cellulose acetate syringe filters before use.

2.2. Equipment and capillary electrophoretic conditions

An ultrasonic bath, 300 Ultrasonik (Ney Company, Bloomfield, USA), was used to sonicate solutions.

A Metrohm 691 pH Meter (Metrohm, Herisau, Switzerland) was used to measure pH.

CE experiments were carried out on a Spectra PHORESIS 1000 (Thermo Separation Products, Fremont, CA, USA) which was driven by CE software (version 3.01) operating under IBM OS/2TM (version 1.2) and contained a programmable high-speed scanning multiple wavelength detector.

The fused (uncoated) silica capillaries were purchased from Supelco (Bellefonte, PA, USA) and had a total length of 44 cm (36 cm to detector), an inner diameter of 50 μm and an outer diameter of 363 μm. Detection wavelength was 240 nm with a rise time of 0.5 s. The detection was towards the cathodic end and a detection window was created by burning off the polyimide coating on the capillary. Hydrodynamic injection was performed for 10 s. The vacuum system of the instrument applied a constant negative pressure of 5.17 kPa for the injection. Capillary temperature was kept at 27 °C and the voltage applied was 18 kV. The standard run buffer consisted of an aqueous solution of 0.10 M boric acid adjusted to pH 8.8 with 1 M NaOH. Under these operating conditions a current of about 18 μA was typically generated. At the beginning of each day, the capillary was rinsed with 0.1 M NaOH for 10 min and then with water for 10 min. To achieve high migration time repeatability, the capillary was treated with 0.1 M NaOH for 2 min before each sample injection, rinsed with Milli-Q water for 2 min and conditioned with BGE for 2 min at 27 °C. Prior to each sequence, two blank injections were performed to stabilize the capillary wall surface and to allow the buffer and the sample solutions to reach a constant temperature on the autosampler tray. The plate numbers were calculated according to the standard expression based on peak width at half height [35].

2.3. Calibration curves

Regression curves were obtained by plotting the analyte/internal standard concentration ratio vs. the peak area/migration time ratio of the analyte divided by the peak area/migration time ratio of the internal standard. The curve for the drug product was evaluated across the 80–120% range of the test concentration (RU 0.02 g l^{-1}), while the linearity for the drug substance was assessed in a wider range, from 5×10^{-4} to 0.15 g l^{-1}. For drug substance, five different concentrations of each analyte were prepared by diluting the standard stock solution with water. For drug product, five separate weighings of synthetic mixtures of the components were used. Each solution was analyzed twice.

2.4. Tablet assay

Twenty coated tablets were weighed and finely powdered. A portion of the powder, accurately weighed and equivalent to about 10 mg of RU, was transferred into a 100 ml volumetric flask. The content was diluted with about 50 ml of water, shaken vigorously, sonicated for 15 min, diluted to volume and filtered through a dry filter, discarding the first portion of the filtrate. A 4 ml portion of the clear filtrate was transferred into a 20 ml volumetric flask to which 4 ml of the internal standard stock solution were added, and the volume was made up to 20 ml with water. Quantification of RU was carried out by means of drug product calibration curves and the generated results were compared with those obtained by an AdSV method [16].

2.5. Experimental design

Experimental design was generated and statistical analysis of experimental data was performed using NEMRODW software package [36]. During the optimization, the experiments of a Doehlert design were carried out in randomized order with RU concentration of 4.9×10^{-5} M and PE concentration of 4.8×10^{-5} M. An eight-run Plackett–Burman matrix was used for robustness testing using a RU concentration of 5.1×10^{-5} M and PE concentration of 4.9×10^{-5} M.

3. Results and discussion

3.1. Method optimization

Setting up of a CZE method for the determina-

tion of an achiral analyte requires consideration of a number of variables to be optimized, such as temperature, voltage, pH and concentration of background electrolyte (BGE) in order to have good efficiency, measured as the number of theoretical plates (N), high sensitivity, measured as peak area/migration time ratio (A/mt), and short analysis time.

At the beginning of experimental work, if the task is easy, it is possible to obtain a partial solution of the problem, to be improved through an optimization. Experimental design can be used to obtain a good description and prevision of the considered problem in order to find the optimum. In particular, through the study of a map (i.e. response surface), faithfully representing the problem, it is possible to identify the conditions yielding the best results.

As for the one-variable-at-a-time approach, the first step of a multivariate optimization concerns the choice of the influential responses and factors. The experiments are then planned in order to homogeneously cover the experimental space for which limits are defined by the researcher.

In this work, preliminary experiments allowed boric acid to be selected as BGE and analysis time, ranging in the 2–4 min interval, could be considered short enough without the need of further optimization. On the contrary, the efficiency and sensitivity of the method were not considered optimal. A good efficiency allows the peak area to be precisely measured and, obviously, a high peak area/migration time ratio allows a low analyte concentration to be measured. Thus, the method was optimized with respect to the number of theoretical plates, and peak area/migration time ratio, both to be maximized. Four variables were involved in the experimental design. As concerns the experimental space investigated, temperature (T, U_1) ranged from 21 to 35 °C; voltage (V, U_2) from 15 to 25 kV; boric acid buffer concentration (BGE conc., U_3) from 0.04 to 0.1 M; pH (pH, U_4) from 8.3 to 8.9. In particular, the experimental domain of each variable was chosen according to preliminary experiments. For example, the range of variation of pH was quite small to avoid the peak tailing effects observed at lower pH and the beginning lack of resolution between RU and PE peaks at higher pH. This choice made it possible to be focused only on the responses A/mt and N of RU, without considering the resolution between analyte and internal standard.

After this first step, the experiments were planned assuming a second-order polynomial relationship between response and factors:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + \varepsilon$$

where y represents the experimental response, x_i the independent evaluated factors, β_0 the intercept, β_i the model coefficients obtainable by multiple regression and ε the experimental error.

The graphical representation of this function is the response surface, which describes the response variation with respect to factor variation. In order to have a true response surface, and thus a true representation of the phenomenon, an accurate estimate of model coefficients has to be performed. These are calculated by multivariate regression, processing the experimental responses obtained in specified points of the experimental domain. Inside this domain, the factors are studied at defined values whose number depends on the selected design. In this case the experiments were planned by a Doehlert design in which the factors are studied at various levels: one at three and one at five, while the remaining k-2 factors at seven levels [33,37]. The researcher can choose the number of levels at which to study a factor depending on the possibility of dividing the experimental domain and as a function of the desired information. In the present case, T was studied at five levels, V and BGE conc. at seven levels and pH at three levels, since the latter was difficult to study at more than three levels, due to its small experimental domain. As concerns the number of experiments, Doehlert design requires $k^2 + k + n$ experiments, where k is the number of variables and n is the number of extra points at the center of the design. The experimental matrix together with the experimental responses is reported in

Table 1
Doehlert design experimental matrix

Number of experiments	U_1	U_2	U_3	U_4	A/mt	N
1	1.0000	0.0000	0.0000	0.0000	2759	95 472
2	−1.0000	0.0000	0.0000	0.0000	2252	116 514
3	0.5000	0.8660	0.0000	0.0000	2666	81 494
4	−0.5000	−0.8660	0.0000	0.0000	2510	121 506
5	0.5000	−0.8660	0.0000	0.0000	2729	113 716
6	−0.5000	0.8660	0.0000	0.0000	2402	95 672
7	0.5000	0.2887	0.8165	0.0000	2802	112 280
8	−0.5000	−0.2887	−0.8165	0.0000	2315	82 647
9	0.5000	−0.2887	−0.8165	0.0000	2596	76 772
10	0.0000	0.5774	−0.8165	0.0000	2278	62 356
11	−0.5000	0.2887	0.8165	0.0000	2561	135 313
12	0.0000	−0.5774	0.8165	0.0000	2661	146 596
13	0.5000	0.2887	0.2041	0.7906	2793	122 683
14	−0.5000	−0.2887	−0.2041	−0.7906	2165	55 457
15	0.5000	−0.2887	−0.2041	−0.7906	2370	60 436
16	0.0000	0.5774	−0.2041	−0.7906	2165	44 183
17	0.0000	0.0000	0.6124	−0.7906	2358	84 269
18	−0.5000	0.2887	0.2041	0.7906	2618	141 462
19	0.0000	−0.5774	0.2041	0.7906	2783	153 366
20	0.0000	0.0000	−0.6124	0.7906	2584	114 476
21	0.0000	0.0000	0.0000	0.0000	2589	109 732
22	0.0000	0.0000	0.0000	0.0000	2522	107 375
23	0.0000	0.0000	0.0000	0.0000	2508	102 563
24	0.0000	0.0000	0.0000	0.0000	2519	98 773

Factors, U_1; temperature, U_2; voltage, U_3; BGE concentration, U_4; pH. Responses, rufloxacin peak area/migration time ratio (A/mt) ; rufloxacin number of theoretical plates (N).

Table 2

Source of variation	Sum of squares	Degrees of freedom	Mean square	F ratio
ANOVA: *rufloxacin peak area/migration time ratio* (A/*mt*)				
Regression	8.68×10^5	14	6.20×10^4	54.55[a]
Residuals	1.02×10^4	9	1.14×10^3	
Lack of fit	6.16×10^3	6	1.03×10^3	0.76[b]
Pure error	4.07×10^3	3	1.36×10^3	
Total	8.78×10^5	23		
Rufloxacin number of theoretical plates (N)				
Regression	1.93×10^{10}	14	1.38×10^9	91.53[c]
Residuals	1.36×10^8	9	1.51×10^7	
Lack of fit	6.34×10^7	6	1.06×10^7	0.44[d]
Pure error	7.21×10^7	3	2.40×10^7	
Total	1.94×10^{10}	23		

[a] $54.55 > F_{\text{crit.}} = 3.07$ (with 12 and 9 degrees of freedom and $\alpha = 0.05$).
[b] $0.76 < F_{\text{crit.}} = 8.94$ (with 6 and 3 degrees of freedom and $\alpha = 0.05$).
[c] $91.53 > F_{\text{crit.}} = 3.07$ (with 12 and 9 degrees of freedom and $\alpha = 0.05$).
[d] $0.44 < F_{\text{crit.}} = 8.94$ (with 6 and 3 degrees of freedom and $\alpha = 0.05$).

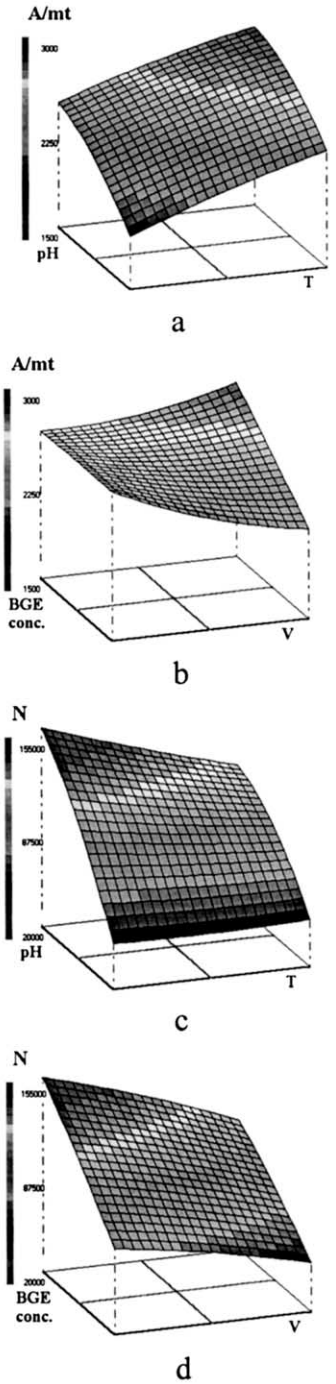

Fig. 1. Rufloxacin peak area/migration time ratio response surfaces obtained by plotting, (a) temperature (T) vs. pH; (b) voltage (V) vs. BGE conc. Rufloxacin number of theoretical plates (N) response surfaces obtained by plotting: (c) temperature (T) vs. pH; (d) voltage (V) vs. BGE conc.

Table 1. At this point the responses were processed by means of the NEMRODW software [36] and the analysis of variance (ANOVA) indicated that the regression models assumed were valid and significant (Table 2) [33,38]. Thus, having found models that faithfully represent the problem, the response surfaces were drawn. In particular the response surfaces were drawn keeping two factors at a time at their central value, the third dimension being represented by the response (Fig. 1). Fig. 1a and b show that to maximize the response A/mt it is necessary to move towards high levels of all considered factors. Fig. 1c and d indicate that, as regards the response N, pH and BGE concentration have to be set at high values. However, the maximization of N was obtained with low values of T and V and this was in conflict with the required conditions to maximize A/mt. A simple way to resolve the problem is to use the Derringer's desirability function (D), with a maximum value of 1. This function is the geometric mean of the partial desirability functions, and starting from its graphical representation it is possible to find the best conditions to simultaneously optimize several responses [33,39,40]. D function and the partial desirability functions d_i, were calculated by means of the NEMRODW software.

The two partial desirability responses, d_1 for A/mt and d_2 for N are reported in Fig. 2. From the D graphs (Fig. 3), obtained setting two factors at their central value, it was possible to see that there was a restricted area where D values were maximum and then fell abruptly to zero. In particular, the optimal conditions chosen were: temperature, 27 °C; voltage, 18 kV; BGE concentration, 0.10 M and pH 8.8. The validation of the models around the optimized conditions was carried out verifying that there was a close agreement between predicted and measured responses. The confidence interval, at a probability level of 99%, was calculated using the standard deviation obtained from the replicates (A/mt: $\bar{X} = 2664$, S.D. = 36.83, $n = 4$; N: $\bar{X} =$ 148315, S.D. = 4904, $n = 4$). The confidence intervals were 2664 ± 108 and $148\,315 \pm 14\,322$ for A/mt and N, respectively. The predicted values (A/mt 2740; N 159 516) were inside the confidence interval.

Applying the optimized conditions, a typical electropherogram was obtained analyzing the drug

product with IS pefloxacin mesylate as reported in Fig. 4.

3.2. Method validation

Validation of this method (i.e. the proof of its suitability for the intended purpose), for the analysis of drug substance and drug product, was assessed following ICH3 guidelines [41]. The performance parameters evaluated were robustness, selectivity, linearity, accuracy and precision. The benefit of using an internal standard to correct errors, which are introduced by variable injection volume, voltage, or EOF, has been reported [42]; thus, to improve precision, an internal standard was used. Pefloxacin mesylate was found to be the best IS within a series of quinolones since it has molecular structure and migration time very close to those of rufloxacin.

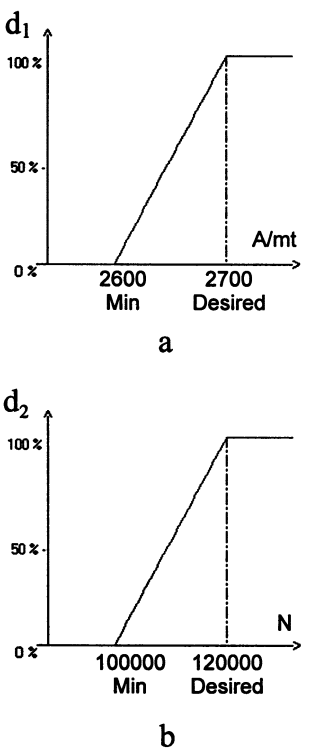

Fig. 2. Transformation of original responses in the individual desirability functions: (a) rufloxacin peak area/migration time ratio response (A/mt); (b) rufloxacin number of theoretical plates response (N).

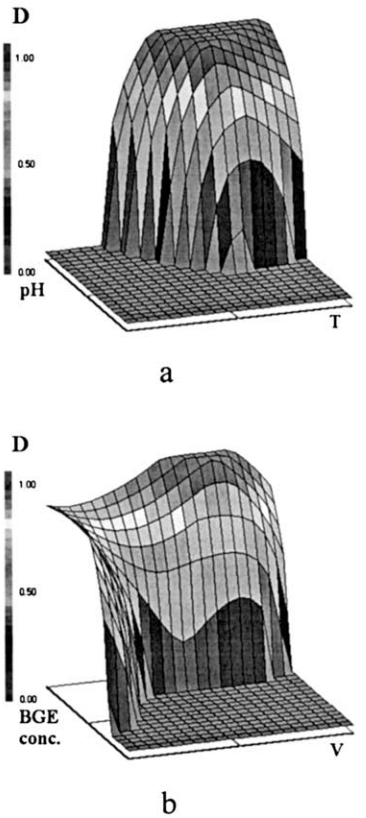

Fig. 3. Three-dimensional plot of desirability function obtained by plotting: (a) temperature (T) vs. pH; (b) voltage (V) vs. BGE conc.

3.2.1. Robustness

Robustness testing examines the potential sources of variability in one or a number of responses considered during the optimization step, according to the aim of the method. To examine the potential sources of variability, a number of factors are selected from the operating procedure and examined in an interval that slightly exceeds the variations expected when a method is transferred between instrument or labs. In general the factor variations can be successfully examined in an experimental design and a Plackett-Burman matrix, where only the main effects are studied, can be very useful for this aim [43]. In fact, during robustness testing, factor interactions can be assumed to be negligible due to the small variations in the factor levels.

In the present case, for the quantitative aim, only the response A/mt was considered in robustness testing among the responses considered in the method optimization. As regards resolution between the two peaks of RU and internal standard PE, even if it is an important response for an assay method, it was not considered during robustness testing. In fact, no resolution problem was observed during the optimization step (resolution was higher than 3.78 in all experiments) where the experimental domain is larger than that used in robustness testing. In particular, the effect of the factors evaluated during the optimization step was taken into consideration but in a smaller experimental domain, defined symmetrically around the optimized values described in the procedure. The explored experimental domain is reported in Table 3; the experimental matrix together with the obtained responses is reported in Table 4.

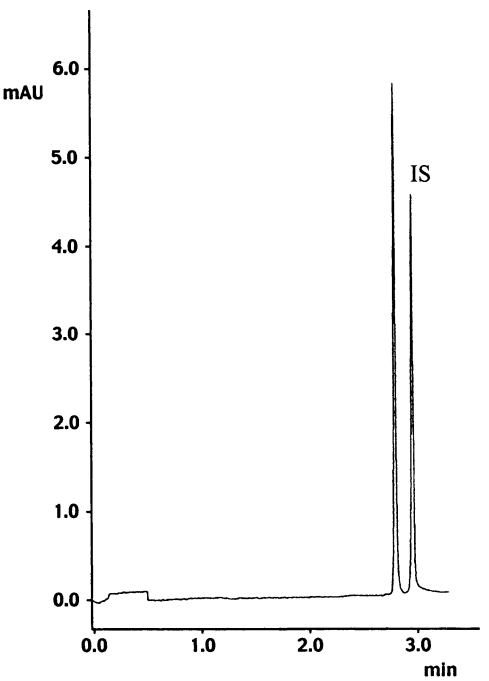

Fig. 4. Typical rufloxacin hydrochloride (drug product) electropherogram using pefloxacin mesylate as internal standard and the following optimized conditions, 0.10 M, pH 8.8 boric acid buffer; voltage 18 kV; temperature 27 °C.

Table 3
Factors and experimental domain in robustness testing

Factor		Experimental domain
U_1	Temperature (°C)	26–28
U_2	Voltage (kV)	17–19
U_3	BGE Concentration (M)	0.09–0.11
U_4	pH	8.7–8.9

Graphic analysis of effects [44] was used to identify the critical factors for method robustness. The advantage of this plot is that the numerical values of the effects are displayed. A bar graph in which the length of each bar is proportional to the absolute effect value, was constructed for the A/mt response (Fig. 5). The effects that exceed the reference lines, corresponding to 95% confidence interval, were those significant for the response. The standard error for the effects was calculated using the three dummy factors available in Plackett-Burman design. It is assumed that the effect of a dummy factor is due to experimental error. Thus the estimated standard error of an effect is given by:

$$(\text{S.E.})_e = \sqrt{\frac{\sum E^2_{\text{dummy};i}}{n_{\text{dummy}}}}$$

where $E_{\text{dummy};i}$ are the effects of the dummy factors and n_{dummy} is the number of dummies. The confidence interval was calculated starting from the estimate of standard error for each coefficient and the value of Student's t for a 95% probability and a number of degrees of freedom, equal to the number of dummies [45].

Temperature (U_1) was found to be the only critical factor for the response A/mt, thus the importance of the experimental conditions of this parameter is pointed out.

3.2.2. Selectivity

The selectivity of the method was assessed. Stability tests under long-term and accelerated storage conditions, as requested by ICH [46], were carried out by the RU drug substance and drug product producer (Mediolanum Farmaceutici, Milan, Italy), demonstrating that degradation products were not formed. Thus, only selectivity towards the tablet excipients was evaluated. A

Table 4
Robustness testing experimental matrix

Number of experiments	U_1	U_2	U_3	U_4	A/mt
1	1	1	1	−1	2784
2	−1	1	1	1	2707
3	−1	−1	1	1	2667
4	1	−1	−1	1	2762
5	−1	1	−1	−1	2692
6	1	−1	1	−1	2733
7	1	1	−1	1	2751
8	−1	−1	−1	−1	2586

Factors, U_1, temperature; U_2, voltage; U_3, BGE concentration; U_4, pH. Response, rufloxacin peak area/migration time ratio (A/mt).

solution of excipients was analyzed according to the method described and an electropherogram, absolutely free of any peak, was obtained.

3.2.3. Linearity

Applying the optimized conditions a linear relationship was found for the drug substance in a 5-level concentration range of 1.2×10^{-6}–3.7×10^{-4} M. The equation found was $y = 1.5699x - 0.0294$ ($n = 5$, $k = 2$) with an R^2 equal to 0.9996 and a cross-validated R^2_{cv} equal to 0.9994.

The curve for drug product was evaluated across the 80–120% range of the test concentration (RU 0.02 g l^{-1}), from 4.0×10^{-5} to 6.0×10^{-5} M. The calibration was limited to this range because it is sufficient to analyze pharmaceutical tablets (that have a RU content of 200 mg). The linear relationship found was $y = 1.2329x - 0.0094$ with an R^2 equal to 0.9986 ($n = 5$, $k = 2$) and an R^2_{cv} equal to 0.9978.

Resolution was always good in the linearity ranges studied (5.10–4.40 for the drug substance and 4.74–4.78 for the drug product).

3.2.4. Accuracy and precision

These parameters were evaluated at three concentration levels covering the linearity range using three replicates. In the case of the drug substance, accuracy was determined by application of the analytical method to the analyte of known purity. In the case of the drug product, accuracy was determined by application of the analytical method to synthetic mixtures of the drug product components to which known amounts of analyte had been added within the method range [41]. Accuracy was measured as percent recovery with the confidence interval ($\alpha/2 = 0.025$) at the low, central and high concentration levels of linearity ranges. For drug substances, the accuracy was 103.3 ± 2.3, 99.2 ± 0.7, 101.4 ± 0.9% and for drug product, using synthetic mixtures, was 97.1 ± 1.4, 98.3 ± 1.7, 99.0 ± 0.2.

The same concentration levels were used to evaluate precision as degree of repeatability performing three replicates. The values of R.S.D. were 0.9, 0.3, 0.4% for the drug substance and 0.6, 0.7, 0.1% for the drug product.

Thus, the obtained results showed that the method was accurate and precise.

Moreover, drug product results generated by CE were compared with those obtained by an AdSV method described by some of us [16] and a good agreement was obtained between the two techniques.

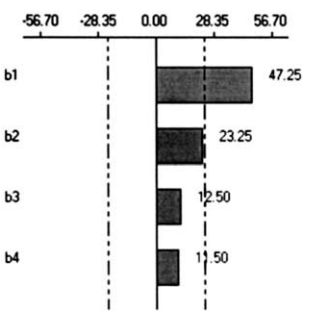

Fig. 5. Rufloxacin peak area/migration time ratio (A/mt) graphic analysis of effects.

4. Conclusions

The multivariate methodology proposed here to set up a CZE method for determining rufloxacin hydrochloride has shown how the design of experiments is advantageous. The optimized and validated method was rapid, selective, reliable and cost effective. Moreover, the use of the response surface methodology allowed a graphical representation of the considered phenomenon to be obtained with a low number of experiments. In addition, the running of statistically designed experiments allowed method robustness to be evaluated. This is an important goal for the industry since robustness is an essential validation parameter, and its correct study is possible only with the use of an experimental design. Thus also for the determination of a single, achiral analyte by means of a separative method, experimental design is a necessary tool.

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