

Optimization of an Electrolyte System for the Simultaneous Separation of Nelfinavir Mesylate and Two Impurities by Micellar Electrokinetic Chromatography

Carina A. Bastos,^a Cláudia R. B. Gomes,^b Marcus V. N. de Souza^b and
Marcone A. L. de Oliveira^{*,a}

^aGrupo de Química Analítica e Quimiometria (GQAQ), Universidade Federal de Juiz de Fora,
36036-900 Juiz de Fora-MG, Brazil

^bFundação Oswaldo Cruz, Instituto de Tecnologia em Fármacos-Far Manguinhos,
21041-250 Rio de Janeiro-RJ, Brazil

A methodology for the simultaneous determination of nelfinavir mesylate and the impurities 3-hydroxy-2-methylbenzoic acid and (2*R*,3*R*)-4-((3*S*,4*aS*,8*aS*)-3-(tert-butylcarbamoil) octahydroisoquinolin-2(1*H*)-yl)-3-hydroxy-1-(phenylthio)butan-2-aminium benzoate by micellar electrokinetic chromatography, with an analysis time of 25 min, was proposed. An electrolyte composed of sodium tetraborate buffer (pH 9.24; 25 mmol L⁻¹), sodium dodecyl sulphate (9 mmol L⁻¹) and methanol (10%, v/v) was optimized using a mixed-level factorial design, with direct detection at 200 nm. After evaluating some figures of merit, such as selectivity, linearity, precision, limit of detection, limit of quantification, accuracy and robustness (using Youden's test), the method was successfully applied to the analysis of nelfinavir mesylate and its impurities in a pharmaceutical formulation. The optimized methodology is demonstrated to be useful in the determination of these analytes in a synthesis monitoring process, in raw materials and in pharmaceutical formulations, while offering low solvent consumption, requiring a small sample and using non-specific columns as advantages.

Keywords: nelfinavir, impurities, micellar electrokinetic chromatography, mixed factorial design, Youden's test

Introduction

Acquired immune deficiency syndrome (AIDS) is an infectious disease caused by human immunodeficiency virus (HIV). HIV differs from other virus because it attacks and damages the immune system. One component of the immune system is the CD4+ T cells that directly attack invading microorganisms. This virus, after infecting the CD4+ T cells, leads to a lack of coordination by the immune system and its progressive ineffectiveness, ultimately establishing an immunodeficiency.¹

The main objective of antiretroviral therapy (TARV) is to slow the progression of immunodeficiency and restore, as much as possible, the normal functions of the immune system, thereby increasing the lifetime and quality of life of the infected person.²

For TARV, there are different drugs that act at different stages of the replication cycle of the virus, including drugs that inhibit the protease, an essential enzyme for virus

maturation.¹ Among protease inhibitors is nelfinavir mesylate ((3*S*,4*aS*,8*aS*)-*N*-(tert-butyl)-2-((2*R*,3*R*)-2-hydroxy-3-(3-hydroxy-2-methylbenzamido)-4-(phenylthio)butyl) decahydroisoquinoline-3-carboxamidemethanesulfonate), the object of this study (Figure 1).

With the expiration of the patent, it is important that other manufacturers are able to produce the drug, so that treatment costs can be reduced. Several synthetic routes are described in the literature for the synthesis of nelfinavir mesylate.³ Among them, the route from *D*-tartaric acid 1 via a reaction of the intermediate cyclic sulphate 6 with potassium phthalimide (Figures 2 and 3) was chosen because of having fewer steps and being economically more viable.

In this synthetic route, the following impurities may be present (Figure 1): 3-hydroxy-2-methylbenzoic acid (impurity A) and (2*R*,3*R*)-4-((3*S*,4*aS*,8*aS*)-3-(tert-butylcarbamoil) octahydroisoquinolin-2(1*H*)-yl)-3-hydroxy-1-(phenylthio)butan-2-aminium benzoate (impurity B).

Among the steps for a drug that can be produced and made available for use is the quality control of the drug,

*e-mail: marcone.oliveira@ufjf.edu.br

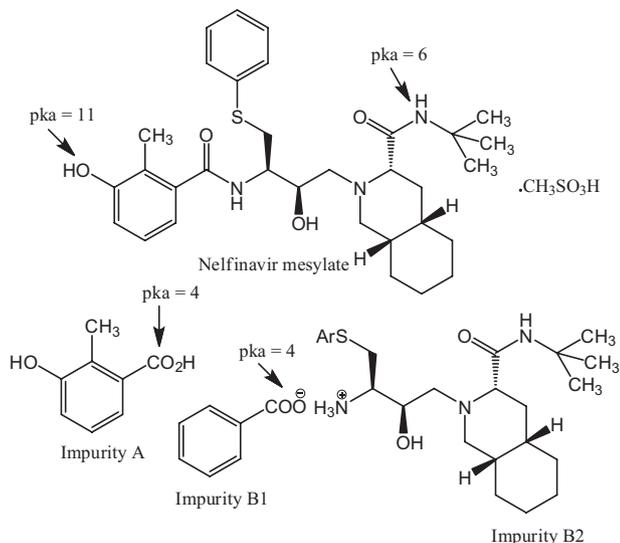


Figure 1. Chemical structures of nelfinavir mesylate and its impurities.

which should contain technical information of the same, as well as specifications assay, impurities tests, and a description of the analytical methodology used, including its validation.⁴

Some methods, such as capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC), can quantify nelfinavir mesylate and are available in the literature.⁵⁻⁹ Seshachalam *et al.*⁵ developed an HPLC method for the quantification of nelfinavir mesylate and five impurities found in the raw materials and pharmaceutical formulations. Jing *et al.*⁶ developed an HPLC method for the determination of nelfinavir mesylate in the presence of its degradation products and three related impurities. However, it is important to emphasize that those literature methodologies were not able to analyze, simultaneously, nelfinavir mesylate and the same impurities described in this work.

CE has been established as a versatile and robust method for providing fast, efficient and automated separations with small amounts of sample, solvent and reagent, thus being more 'eco-friendly' than HPLC.^{10,11} Micellar electrokinetic chromatography (MEKC), a CE mode, makes the separation of neutral compounds possible for use in different applications.¹² This study proposed to develop a method for the simultaneous determination of nelfinavir mesylate and its impurities using MEKC, which included the optimization of the analysis method using the design of experiments, the validation of the proposed analytical methodology and its application in a real sample.

Experimental

Material

Chemicals and reagents

All chemicals were of analytical grade. Sodium dodecyl sulphate (SDS), sodium tetraborate decahydrate and Sudan III were purchased from Vetec (Rio de Janeiro, Brazil). Sodium hydroxide (NaOH) was purchased from Labsynth Produtos para Laboratórios Ltda (São Paulo, Brazil). Methanol was purchased from Vetec (Rio de Janeiro, Brazil) and Dinâmica Química Contemporânea Ltda (São Paulo, Brazil). The standards and samples were provided by the Fundação Oswaldo Cruz, Instituto de Tecnologia em Fármacos - Far Manguinhos (Rio de Janeiro, Brazil).

A stock aqueous solution of sodium tetraborate buffer (TB) (pH 9.24, 100 mmol L⁻¹) and a stock aqueous solution of SDS (100 mmol L⁻¹) were used for the preparation of the electrolytes. Amounts of the standards (1,000 mg L⁻¹) were separately prepared in methanol (MeOH) and stored at 2-3 °C.

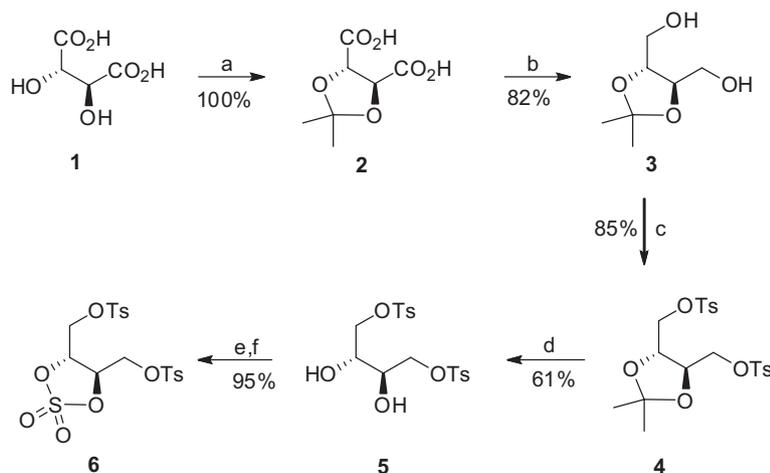


Figure 2. Synthesis of intermediate cyclic sulphate 6. (a) DMP/*p*-TsOH/MeOH; (b) NaBH₄/EtOH; (c) *p*-TsCl/NEt₃/MTBE; (d) EtOH/1N HCl, reflux; (e) SOCl₂/CH₂Cl₂/r.t., 18 h; (f) cat. RuCl₃/NaIO₄/CH₃CN/H₂O.

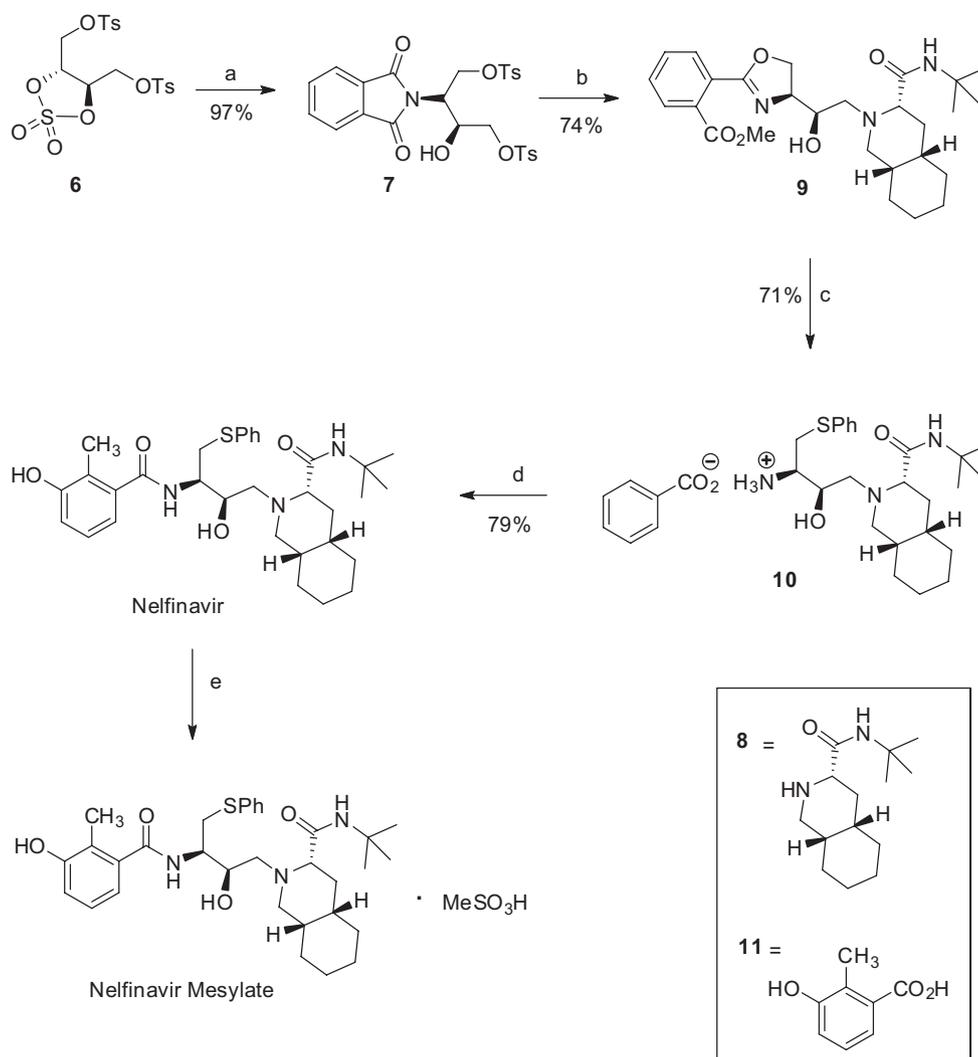


Figure 3. Synthesis of nelfinavir mesylate. (a) PhThNK/H₂SO₄; (b) **8**/K₂CO₃/CH₃CN/MeOH; (c) (1) PhSH/KHCO₃/THF; (2) ethanolamine/benzoic acid; (d) (1) **11**/EtOH; (2) NaOH; (e) MeOH/CH₂Cl₂/MeSO₃H.

Instrumentation

The experiments were conducted in a CE system (CE-7100; Agilent Technologies, Palo Alto, USA) equipped with a diode array detector (DAD) set at 200 nm, a temperature control device maintained at 20 °C and data acquisition and treatment software (HP ChemStation, ref A.06.01). Samples were hydrodynamically injected (50 mbar 3 s) and the electrophoretic system was operated under normal polarity and constant voltage conditions (+20 kV). Peak width > 0.05 min (1.0 s response time) (5 Hz) was used. For all experiments, a 48.5 cm (40.0 cm effective length) × 50 μm (inner diameter) fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used.

Methods

Standard preparation

The method was optimized using the same concentration

level for all the compounds, after dilution of the stock solution in methanol.

For the validation procedures, the concentrations were adjusted in order that the impurities represented 0.5% of the drug concentration and, in this way, two solutions were used: (i) solution 1: containing 100 mg L⁻¹ of impurity A; 100 mg L⁻¹ of impurity B; and 20,000 mg L⁻¹ of nelfinavir mesylate, for impurities quantification; (ii) solution 2: containing 100 mg L⁻¹ of nelfinavir mesylate, for drug quantification.

Sample preparation

Twenty tablets were weighed and ground to homogeneously fine powders. The powder, corresponding to 250 mg of nelfinavir mesylate (as given by the manufacturer), was weighed and dissolved with 2 mL of methanol in a separate volumetric flask. After 10 min of sonication, the solution was filtered through a 0.45 μm

millipore filter in order to obtain a clear solution. For the determination of analytes, two solutions were injected: (i) for impurities quantification: the filtered solution, with a final concentration equal to 20,000 mg L⁻¹ of nelfinavir mesylate; (ii) for drug quantification: 25 µL of the filtered solution was transferred to a 5 mL volumetric flask and diluted with methanol to a final concentration equal to 100 mg L⁻¹ of nelfinavir mesylate.

Analytical procedures

Conditioning of new capillaries was carried out by a pressure flush of a NaOH solution (1 mol L⁻¹) (30 min), deionized water (15 min) and electrolyte solution (15 min). Between runs, replenishment of the vials containing electrolyte was performed and the capillary was conditioned with a NaOH (0.2 mol L⁻¹) solution (2 min), ultrapure water (2 min) and fresh electrolyte solution (3 min). At the end, the capillary was conditioned with a NaOH solution (1 mol L⁻¹) (5 min) and ultrapure water (5 min).

External standard curves

External standard curves in triplicate were prepared for the analytes by diluting the standard solutions in methanol. The calibration curve levels were: 80, 90, 100, 110 and 120 mg L⁻¹.

Migration time ratio (t_0/t_{mc})

A flow marker, methanol, was injected in order to obtain t_0 and a neutral marker, Sudan III, was injected in order to obtain t_{mc} for the Youden's test.

Results and Discussion

Wavelength selection

The wavelength selection was based on the electronic spectrum of each analyte (Figure 4), dissolved in methanol at a concentration of 250 mg L⁻¹ and injected in the CE system using optimized conditions. The selection of 200 nm simultaneously took into account the following features: wavelength of the highest molar absorptivity and the baseline stability present in the electropherograms. It is important to highlight that all spectra were obtained through a DAD detector in the CE equipment taking into account the background electrolyte (BGE) as the solvent.

Preliminary study

An elegant way to perform the optimization in CE is through use of an effective mobility curve (μ_{eff}). This plot gives information about the mobility of the analytes as a function of the pH. In other words, it is possible to theoretically select the pH range that can achieve the separation of the compounds of interest when the analyte mobilities are distinct. Thus, in the present case, through the μ_{eff} plot in Figure 5, it is possible to see that the simultaneous separation of nelfinavir mesylate, impurities A, B1 and B2 (Figure 1) is very hard with conventional capillary zone electrophoresis, since each has a very similar mobility with at least one of the other compounds. Within this context, due to the behaviour

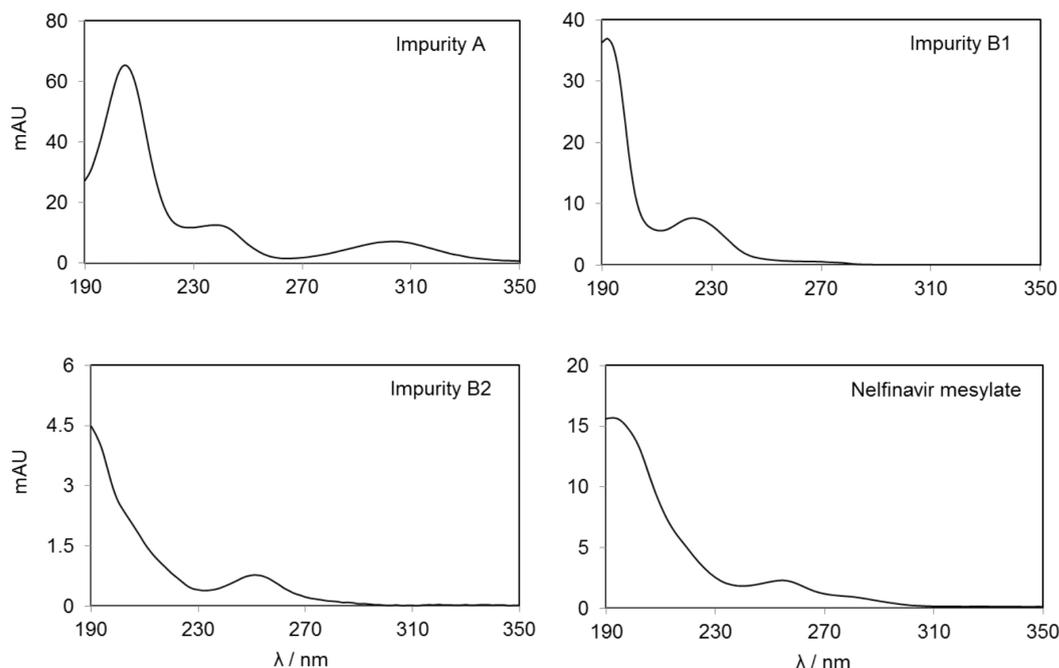


Figure 4. Electronic spectra of nelfinavir mesylate and impurities A, B1 and B2 obtained through a DAD detector in the CE equipment.

of the analytes of interest, MEKC was selected as the methodology to be investigated.

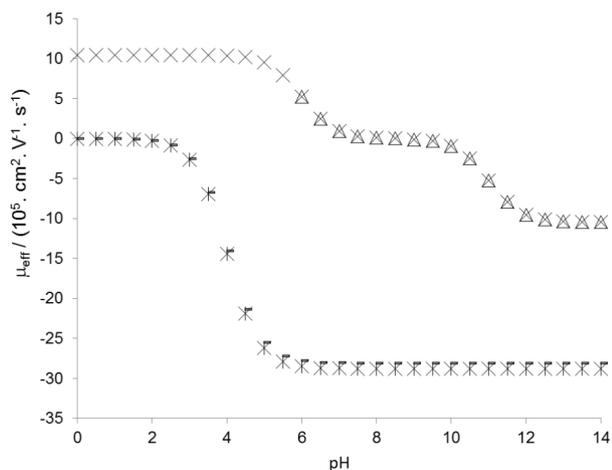


Figure 5. Effective mobility curves of nelfinavir mesylate (x), impurity A (-), impurity B1 (*) and impurity B2 (Δ).

In order to perform a preliminary investigation, the BGE consisted of TB (pH 9.24, 25 mmol L⁻¹) and SDS (25 mmol L⁻¹) and the dilutions of the analytes in MeOH were initially tested. The BGE concentration initially tested took into account the need to avoid bubble formation (in the case of the high SDS concentration) and high current (due to the TB concentration). From a visual analysis of the electropherogram (not shown) it was observed that impurities A and B1 presented similar electrophoretic mobilities, but did not co-elute for the standard mixture tested. On the other hand, elution of nelfinavir mesylate resulted in considerable band broadening and co-elution with impurity B2.

Due to the complex mechanisms and variables involved in MEKC separations, experimental design signal is

an interesting tool for the optimization of separation conditions in comparison with univariate methodologies.¹³ In order to investigate the simultaneous separation of these compounds by MEKC, a ‘design of experiments’ approach was considered. Thus, the variables TB, SDS and MeOH were evaluated through a mixed-level factorial design (MFD), in order to optimize a BGE composition able to achieve simultaneous separation of nelfinavir mesylate, impurities A, B1 and B2. MFD, in spite of being unusual, can be very interesting because it permits the selection of a different range of levels, thereby increasing the experimental information obtained. In the present case, it was necessary to use a MFD because the number of levels for each factor was different, that is 2, 5 and 3 for TB, SDS and MeOH respectively, which resulted in a MFD of type 2 × 5 × 3, totalling 30 experiments. It is important to remember that the level values used were based on the preliminary investigation. Several aspects of the factors selection can be highlighted: TB is usually selected as the buffer of choice in the MEKC approach, since its solubility limit results in a pH of about 9.00, which is an interesting separation strategy, promotes a high electroosmotic flow (EOF), and increases the efficiency while decreasing the analysis time; SDS is a common anionic component for MEKC and, more generally, in pharmaceutical separations gives good results; finally, the addition of organic solvents changes the viscosity and dielectric constant of the BGE and, consequently, the zeta potential of the capillary, resulting in a variation of the electroosmotic flow.¹⁴ Other operational conditions such as injection time, wavelength, cartridge temperature and applied voltage were kept fixed, as described in the experimental part. Table 1 shows the coded matrix, factors and levels used to perform the experiments.

Table 1. Mixed-level factorial design matrix containing factors and levels

Experiment	X ₁	X ₂	X ₃	Experiment	X ₁	X ₂	X ₃
1	-1	-1	-1	16	1	-1	-1
2	-1	-0.5	-1	17	1	-0.5	-1
3	-1	0	-1	18	1	0	-1
4	-1	0.5	-1	19	1	0.5	-1
5	-1	1	-1	20	1	1	-1
6	-1	-1	0	21	1	-1	0
7	-1	-0.5	0	22	1	-0.5	0
8	-1	0	0	23	1	0	0
9	-1	0.5	0	24	1	0.5	0
10	-1	1	0	25	1	1	0
11	-1	-1	1	26	1	-1	1
12	-1	-0.5	1	27	1	-0.5	1
13	-1	0	1	28	1	0	1
14	-1	0.5	1	29	1	0.5	1
15	-1	1	1	30	1	1	1

X₁: TB (mmol L⁻¹): (-1) 15; (1) 25; X₂: SDS (mmol L⁻¹): (-1) 10; (-0.5) 20; (0) 30; (0.5) 40; (1) 50; X₃: MeOH (% v/v): (-1) 0; (0) 5; (1) 10.

In this work, it was not performed replicate in the central point, because the response was evaluated qualitatively, taking into account the electrophoretic profile obtained for

each experimental trial carried out. The experiments were performed randomly and the electropherograms obtained are shown in Figures 6 and 7.

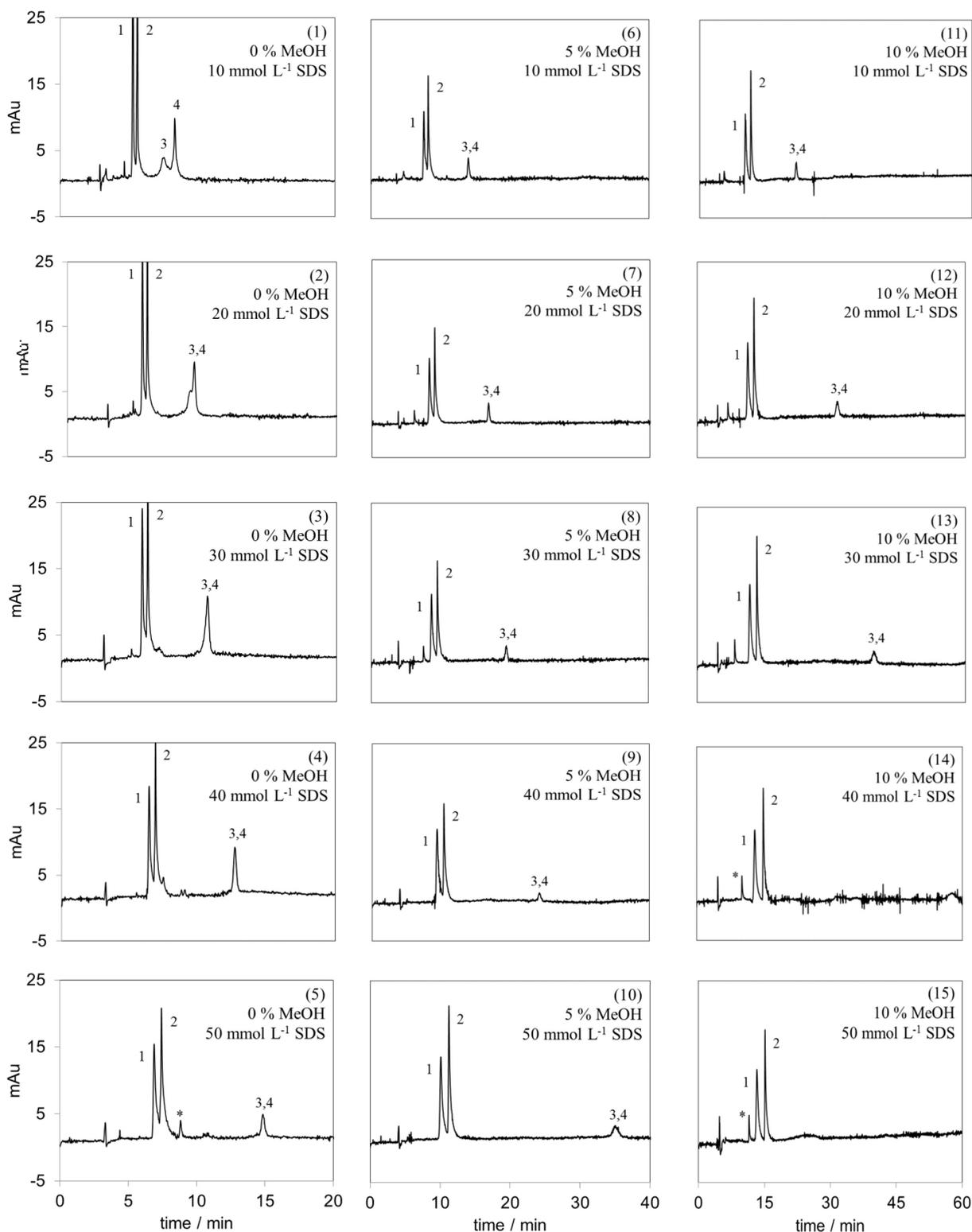


Figure 6. Electropherogram of standards mixture: (1) impurity A, (2) impurity B1, (3) impurity B2, (4) nelfinavir mesylate. Experimental conditions: electrolyte, TB (pH 9.24; 15 mmol L⁻¹); cartridge temperature, 20 °C; injection, 50 mbar 3 s; voltage, +20 kV; λ , 200 nm; capillary, 50 μ m \times 48.5 cm (40.0 cm effective length).

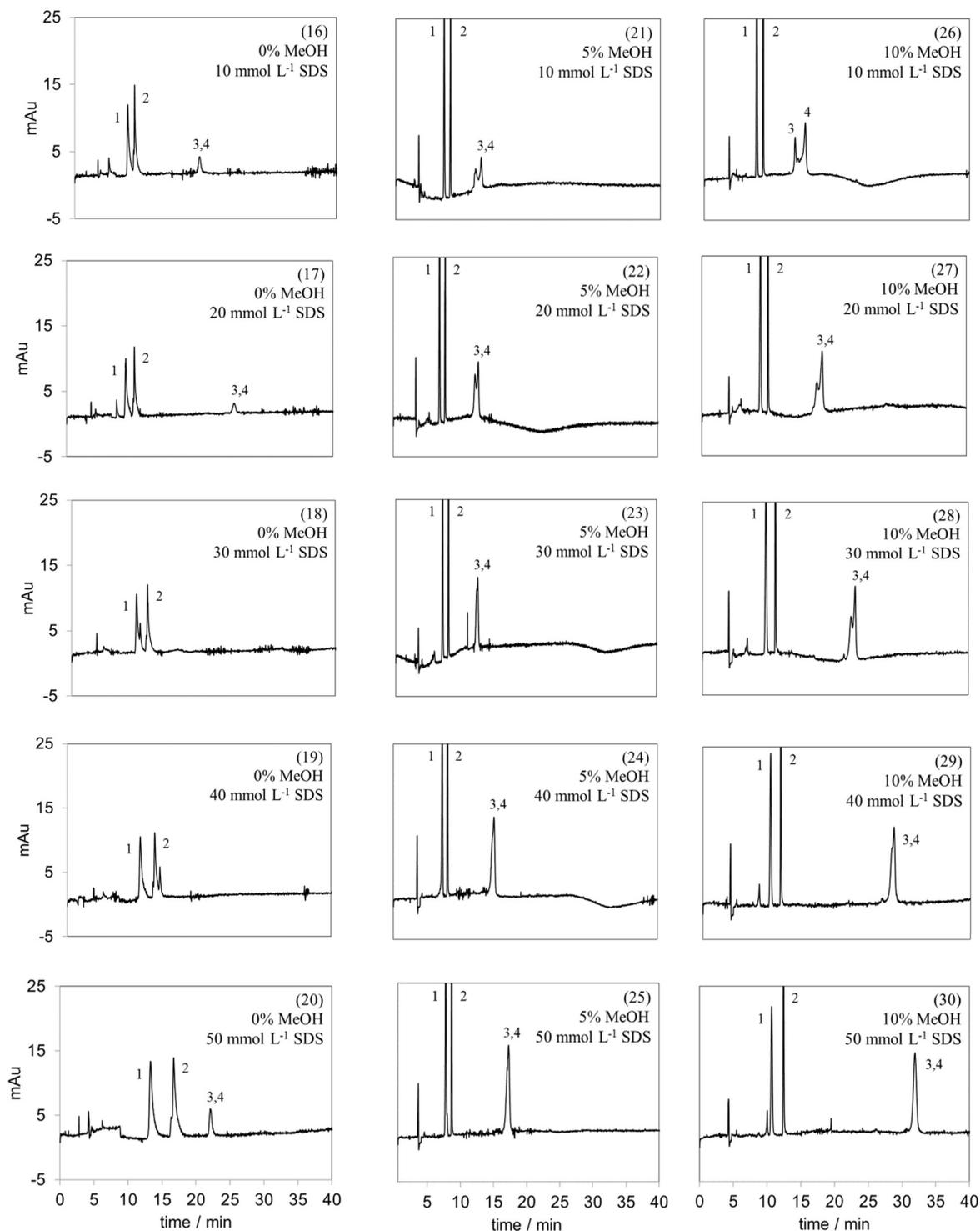


Figure 7. Electropherogram of standards mixture: (1) impurity A, (2) impurity B1, (3) impurity B2, (4) nelfinavir mesylate. Experimental conditions were described in Figure 6.

According to Nishi and Terabe,¹⁵ in MEKC the resolution (R_s) is given by:

$$R_s = \frac{N^{1/2}}{4} \frac{(\alpha - 1)}{(\alpha)} \frac{(k'_2)}{(1 + k'_2)} \frac{\left(1 - \frac{t_0}{t_{mc}}\right)}{\left(1 + \left(\frac{t_0}{t_{mc}}\right) k'_1\right)} \quad (1)$$

where $\alpha = k'_2/k'_1$ is the separation factor.

For neutral analytes, the migration time (t_R) is limited to being between the migration time of the electroosmotic flow (t_0) and that of the micelle (t_{mc}), and the capacity factor k' of the analyte can be calculated by:

$$k' = \frac{t_R - t_0}{t_0(1 - t_R/t_{mc})} \quad (2)$$

On the other hand, the capacity factor k' for an acidic analyte and an anionic micelle, where ion pairing between the two is absent, is given by:

$$k' = \frac{\mu_{ep,S^*} - \mu_{ep,S}}{\mu_{ep,mc} - \mu_{ep,S^*}} \quad (3)$$

where $\mu_{ep,S}$ is the electrophoretic mobility of the analyte in the absence of the micelle and μ_{ep,S^*} is the electrophoretic mobility of the analyte in the presence of the micelle.

So, to evaluate the two critical peaks pairs quantitatively (the first pair consisting of the acidic analytes and the second pair consisting of the neutral analytes), rigorously, it would be necessary to inject a flow marker, such as methanol, for each one of the 30 experiments in order to obtain t_0 , and a neutral marker, such as Sudan III, in order to obtain t_{mc} . Furthermore, it would be necessary to also repeat the 30 experiments using electrolytes without micelle, in order to obtain $\mu_{ep,S}$. Thus, taking into account the above discussion, a total of ninety experiments would be necessary to get the necessary information to calculate the resolution. Once the aim of the experimental design is to obtain the best conditions with a reduced number of experiments, it was decided to perform a qualitative evaluation of the separation profile from the experimental setup using just the 30 experiments.

Thus, the 30 electropherograms obtained were separated into two groups: one performed with 15 mmol L⁻¹ of TB (Figure 6) and the other performed with 25 mmol L⁻¹ of TB (Figure 7). Taking into account the first group (experiments 1 to 15), the profile separation of the critical pair of peaks 1 and 2 practically did not change when the MeOH concentration was fixed at each one of the 3 levels, independent of the SDS concentration. On the other hand, the critical pair of peaks 3 and 4 just signalled a beginning of the separation in experiment 1, which used 0% MeOH and 10 mmol L⁻¹ of SDS. Taking into account the second group (experiments 16 to 30), although the critical pair of peaks 1 and 2 had acceptable separation in all of experiments, the critical pair of peaks 3 and 4 had a total or partial co-elution. It is important to observe that an improvement in the separation profile between peaks 3 and 4 occurred when the MeOH concentration was fixed at a high level (10%) and the SDS concentration was decreased (experiments 30, 29, 28, 27 and 26). In this way, a univariate test was performed in order to evaluate the SDS concentrations at levels lower than 10 mmol L⁻¹, i.e., 9 and 8 mmol L⁻¹. Considering that in a real sample the concentration of the nelfinavir mesylate,

peak 4, will be present at a 200 fold higher concentration in comparison to the impurity B2, peak 3 (Figure 9), the best separation profile observed used 9 mmol L⁻¹ of SDS in the BGE as shown in Figure 8.

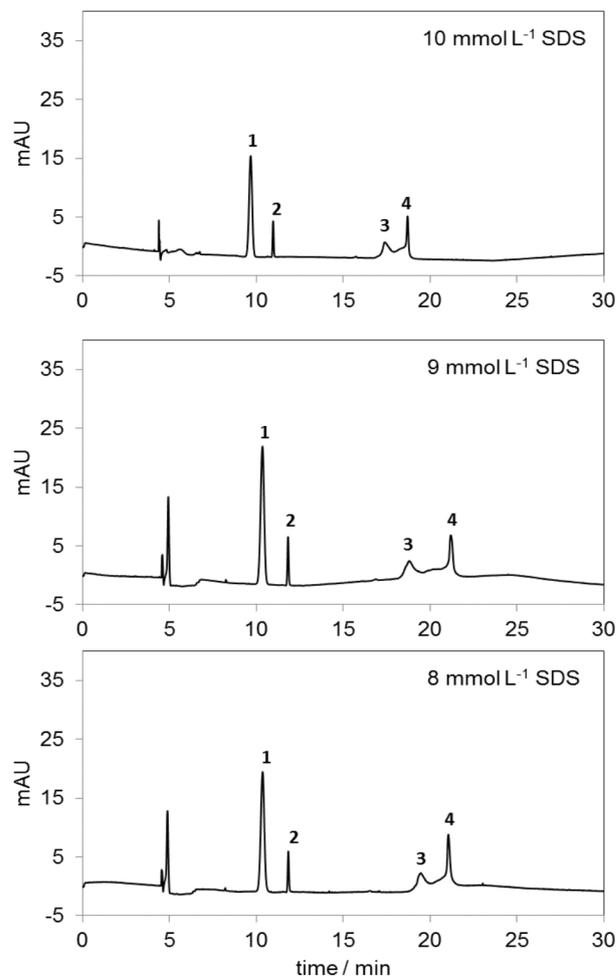


Figure 8. Electropherogram of standards mixture: 100 mg L⁻¹ (1) impurity A, (2) impurity B1, (3) impurity B2, (4) nelfinavir mesylate. Experimental conditions: electrolyte, TB (pH 9.24; 25 mmol L⁻¹) and MeOH (10%, v/v); cartridge temperature, 20 °C; injection, 50 mbar 3 s; voltage, +20 kV; λ , 200 nm; capillary, 50 μ m \times 48.5 cm (40.0 cm effective length).

Instead of preparing standard solutions in MeOH, a dilution of the stock standard solutions in SDS (9 mmol L⁻¹) was tested, but nelfinavir mesylate (peak 4) and impurity B2 (peak 3) co-eluted again, showing that the interaction between both analytes and SDS must happen only during the run, otherwise the separation is impaired. Another test was performed with a greater concentration of MeOH in the electrolyte, which presented a better profile separation between peaks 3 and 4, but this caused a considerable increase in noise.

On the presented electropherograms, the peak related to nelfinavir has a distorted shape. Although the addition of

MeOH seems to increase the peak broadening, its addition is necessary to obtain a separation between peaks 3 and 4. So, in order to improve the baseline, disturb presented to peak 4 and decrease the analysis time, exhaustive tests varying the voltage, the injection time and increasing the temperature or decreasing the capillary length were performed. Just as in the conditions described in Figure 7, it was possible to obtain an acceptable separation between peaks 3 and 4, with an analytical frequency of two samples *per* hour. However, it is important to stress that the baseline behaviour was not critical for analyte quantification in the real sample, as will be demonstrated in the validation section. Finally, before the validation procedures, the final concentrations of the analytes were changed in order that the impurities represented 0.5% of the drug concentration (Figure 9).

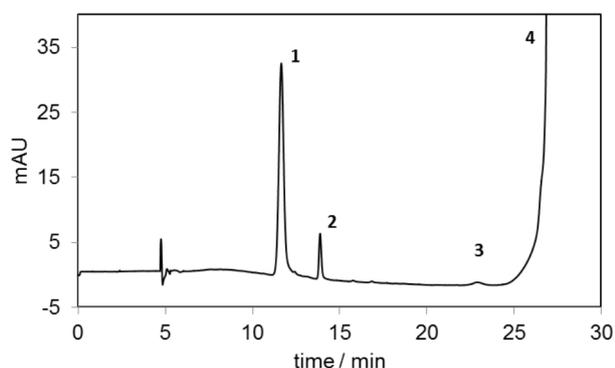


Figure 9. Electropherogram of standards containing 100 mg L⁻¹ of impurities A and B; and 20000 mg L⁻¹ of nelfinavir mesylate: (1) impurity A, (2) impurity B1, (3) impurity B2, (4) nelfinavir mesylate. Experimental conditions: electrolyte, TB (pH 9.24; 25 mmol L⁻¹), SDS (9 mmol L⁻¹) and MeOH (10%, v/v); cartridge temperature, 20 °C; injection, 50 mbar 3 s; voltage, +20 kV; λ , 200 nm; capillary, 50 μ m \times 48.5 cm (40.0 cm effective length).

Validation procedures

After optimization of the electrophoretic conditions, some validation parameters, such as selectivity, linearity, precision, limit of detection (LOD), limit of quantification (LOQ), accuracy and robustness, were determined according to Resolution ANVISA RE No. 899, of 29/05/2003 and ICH.^{16,17}

Table 2. Results obtained from the linearity study

	Slope	Intercept	ANOVA ^a	Residue normality ^b	Homoscedasticity ^c
Nelfinavir mesylate	1.04 (\pm 0.0337)	1.78 (\pm 3.41)	0.848	0.610	0.356
Impurity A	7.15 (\pm 0.0985)	-5.01 (\pm 9.95)	0.752	0.623	0.371
Impurity B1	0.679 (\pm 0.0221)	0.627 (\pm 2.23)	0.111	0.783	0.405
Impurity B2	0.809 (\pm 0.0266)	-2.37 (\pm 2.69)	0.192	0.394	0.376

n = 3 (genuine replicates); range (mg L⁻¹): 80-120; ^aANOVA *p*-value, significance level *p* < 0.05; ^bresidue normality test *p*-value (Shapiro-Wilk test), significance level *p* < 0.05; ^cresidue homogeneity test (Cochran test), critical value = 0.684, significance level of 0.05.

Selectivity and linearity

Method selectivity was assessed by the peak purity test (comparison between the analyte peak and the auto threshold in the purity plot) using the diode array detector. The analyte electrophoretic peak was not found to be attributable to more than one component.^{16,17}

The linearity test was conducted by plotting three standard curves, in order to assess the linear relationship between the concentration of the analyte and the obtained areas. For this purpose, the data for each concentration range, after fitting with an ordinary least squares method, were statistically evaluated taking into account homoscedasticity (Cochran's test), residues' normality (Shapiro-Wilk's test) and lack of fit test (ANOVA) (Table 2).

Precision, limit of detection (LOD) and limit of quantification (LOQ)

Precision can be determined through the estimate of the relative standard deviation (RSD).¹⁶ The precision in the validation of this optimized method was performed at a repeatability level. Repeatability (n = 3) in the sample area was carried out for 80, 90, 100, 110 and 120% of the test concentration. In the present case, concentrations of 80, 90 and 100 mg L⁻¹ of nelfinavir mesylate and the impurities were used. All results presented acceptable precision values (not exceeding 5.00%) as shown in Table 3.

LOD and LOQ were calculated based on the standard deviation of the response and the slope.¹⁶ The LOD and LOQ obtained presented acceptable values as presented in Table 3.

Accuracy

Accuracy, in the present case, was calculated as the percentage of the recovery by the assay of a known added amount of analyte in the sample at five levels of concentrations: 80, 90, 100, 110 and 120 mg L⁻¹.^{16,17} The obtained results showed that the method presents an acceptable accuracy within a 95% confidence interval.

Table 3. RSD (%) obtained from repeatability. LOD and LOQ values

	Nelfinavir mesylate	Impurity A	Impurity B1	Impurity B2
Repeatability	2.82 ^a	1.13 ^a	2.33 ^a	1.96 ^a
	3.89 ^b	1.71 ^b	1.14 ^b	3.68 ^b
LOD / (mg L ⁻¹)	14.7	12.8	8.03	18.7
LOQ / (mg L ⁻¹)	49.0	42.6	26.8	62.3

^aConcentration found; ^bmigration time.

Robustness

The robustness was performed using Youden's test.¹⁸ Seven analytical parameters were selected and small variations were induced in the nominal values of the method (Table 4).

The analytical conditions at the nominal values are represented by capital letters and the conditions with the small variation are represented by lowercase letters. The seven parameters and the respective variations were combined into eight assays or electrophoretic runs, performed in a random order. Table 5 demonstrates the factorial combination of the parameters for the Youden's test.

The analyses results are shown by letters from s to z. Hence, when combination 1 was assayed, the obtained

result was s. When combination 2 was assayed, the obtained result was t, and so on. The results obtained in the eight runs are demonstrated in Table 6.

In Table 6, besides the migration times, it can be seen the separation factor (α). To evaluate the effect of each parameter in Table 6, the average of the four values corresponding to the altered conditions was subtracted from the average of the four values obtained at the nominal conditions. The effects of the parameter variations in the analysis results are presented in Table 7.

The migration time of the last peak (t_{m_4}) was considerably more influenced by one analytical parameter. Thus, a decrease of 2% (v/v) in the MeOH concentration in the BGE induced a media reduction of 3.51 min in t_{m_4} . The migration time ratio, t_0/t_{mc} , is directly related to the

Table 4. Analytical parameters and variations for the robustness evaluation

Parameter	Nominal condition			Variation	
Buffer concentration in electrolyte / (mmol L ⁻¹)	25	A		23	a
SDS concentration in electrolyte / (mmol L ⁻¹)	9	B		11	b
Methanol concentration in electrolyte / (% v/v)	10	C		8	c
Cartridge temperature / °C	20	D		22	d
Voltage condition / kV	20	E		18	e
Injection condition / s	3	F		4	f
Methanol supplier	X	G		Y	g

Table 5. Factorial combination of the analytical parameters for robustness evaluation by Youden's test

Analytical parameter	Factorial combination							
	1	2	3	4	5	6	7	8
Buffer concentration	A	A	A	A	a	a	a	a
SDS concentration	B	B	b	b	B	B	b	b
Methanol concentration	C	c	C	c	C	c	C	c
Cartridge temperature	D	D	d	d	d	d	D	D
Voltage condition	E	e	E	e	e	E	e	E
Injection condition	F	f	f	F	F	f	f	F
Methanol supplier	G	g	g	G	g	G	G	g
Result	s	t	u	v	w	x	y	z

Table 6. Results obtained in eight runs performed for robustness evaluation

Condition	Migration time / min					Separation factor
	tm ₁	tm ₂	tm ₃	tm ₄	t ₀ /t _{mc}	
1	9.96	11.5	18.2	22.3	0.187	3.92
2	9.86	11.3	15.1	20.6	0.173	2.77
3	9.48	10.9	14.1	21.1	0.153	3.38
4	9.80	11.1	16.6	20.5	0.210	4.65
5	10.6	12.1	17.3	22.2	0.206	5.93
6	8.07	9.05	10.9	15.8	0.235	8.27
7	10.8	12.4	16.1	23.0	0.205	8.43
8	8.87	10.0	14.4	17.7	0.225	4.90

Table 7. Effects of the analytical parameters in migration time, migration time ratio (t₀/t_{mc}) and separation factor (α) of the proposed method

Effect	tm ₄ / min ^a	t ₀ /t _{mc} ^a	α _{3,4} ^a
Buffer concentration / (mmol L ⁻¹); (A = 25; a = 23)	1.45	-0.037	-3.20
SDS concentration / (mmol L ⁻¹); (B = 9; b = 11)	-0.382	0.002	-0.116
Methanol concentration / %; (C = 10; c = 8)	3.51	-0.023	0.269
Cartridge temperature / °C; (D = 20; d = 22)	1.00	-0.004	-0.553
Voltage condition / kV; (E = 20; e = 18)	-2.35	0.002	-0.325
Injection condition / s; (F = 3; f = 4)	0.532	0.016	-0.859
Methanol supplier; (G = X; g = Y)	-0.021	0.020	2.07

^aAverage of the values obtained at nominal conditions – average of the values obtained at altered conditions.

width of the migration time window. The smaller the value of t₀/t_{mc}, the wider the migration time window and hence the higher the resolution value. It is necessary to reduce the velocity of the electroosmotic flow to obtain a smaller value of t₀/t_{mc}. Addition of an organic solvent, such as MeOH, is a useful method, however, in practice, a longer run time is required¹⁵ and this was observed. The analytical parameter with the greatest effect on the value of t₀/t_{mc} and on the separation factor (α_{3,4}) was the buffer concentration in the electrolyte. With a reduction of 2 mmol L⁻¹ in the buffer concentration, there was an average increase of 0.037 in t₀/t_{mc} and 3.20 in α_{3,4}. Some parameters such as SDS concentration, cartridge temperature and injection conditions exhibited little influence on the evaluated factors of the electrophoretic method.

Quantitative determination in pharmaceutical formulation (tablets)

After evaluating validation parameters, the optimized method was applied to the quantification of a pharmaceutical formulation with a declared content of 250 mg of nelfinavir mesylate, obtaining 239 mg (± 1.26%) of nelfinavir mesylate as a result. Other compounds were not detected (Figure 10).

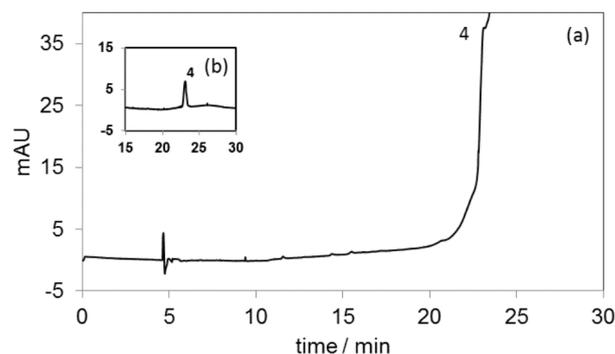


Figure 10. Electropherogram of sample (a) containing 20,000 mg L⁻¹ of nelfinavir mesilate (4) and (b) containing 100 mg L⁻¹ of nelfinavir mesilate (4). Experimental conditions were described in Figure 9.

Conclusion

A methodology using MEKC, capable of simultaneously analyzing nelfinavir and the impurities coming from a synthetic route, was optimized using a MFD, which is demonstrated to be a very interesting tool to help the optimization in CE, despite being little used. The results demonstrated that the optimized methodology can be useful for the determination of these analytes in a synthesis monitoring process, raw materials and pharmaceutical

formulations, as well as offering acceptable efficiency, LOQ, low solvent consumption (eco-friendly), small amount of sample and the use of non-specific columns as advantages.

Supplementary Information

Synthesis and characterization of the standards are available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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References

1. Souza, M. V. N. D.; Almeida, M. V. D.; *Quím. Nova* **2003**, *26*, 366.
2. Silva, G. R. D.; Condessa, F. A.; Pianetti, G. A.; Nunan, E. D. A.; Campos, L. M. M. D.; *Quím. Nova* **2008**, *31*, 1686.
3. Albizati, K. K.; Babu, S.; Birchler, A.; Busse, J. K.; Fugett, M.; Grubbs, A.; Haddach, A.; Pagan, M.; Potts, B.; Remarchuk, T.; Rieger, D.; Rodriguez, R.; Shanley, J.; Szendroi, R.; Tibbetts, T.; Whintten, K.; Borer, B. C.; *Tetrahedron Lett.* **2001**, *42*, 6481.
4. ftp://ftp.saude.sp.gov.br/ftpsessp/bibliote/informe_eletronico/2009/iels.nov.09/iels214/U_RS-MS-ANVISA-RDC-57_171109.pdf accessed in June 2014.
5. Seshachalam, U.; Rajababu, B.; Haribabu, B.; Handrasekhar, K. B.; *J. Liq. Chromatogr. Relat. Technol.* **2008**, *31*, 395.
6. Jing, Q.; Shen, Y.; Tang, Y.; Ren, F.; Yu, X.; Hou, Z.; *J. Pharm. Biomed. Anal.* **2006**, *41*, 1065.
7. Gutleben, W.; Tuan, N. D.; Stoiber, H.; Dierich, M. P.; Sarcletti, M.; Zemmann, A.; *J. Chromatogr. A* **2001**, *922*, 313.
8. Pereira, E. A.; Micke, G. A.; Tavares, M. F. M.; *J. Chromatogr. A* **2005**, *1091*, 169.
9. Tavares, M. F. M.; Jager, A. V.; Silva, C. L. D.; Moraes, E. P.; Pereira, E. A.; Lima, E. C. D.; Fonseca, F. N.; Tonin, F. G.; Micke, G. A.; Santos, M. R.; Oliveira, M. A. L. D.; Moraes, M. D. L. L. D.; Kampen, M. H. V.; Fujiya, N. M.; *J. Braz. Chem. Soc.* **2003**, *14*, 281.
10. Zhang, H.; Liang, F.; Feng, X.; Zhang, H.; Yua, A.; Fei, Q.; *Anal. Methods* **2014**, *6*, 725.
11. Monzón, C. M.; Vera-Candiotti, L.; Sarno, M. D. C.; Delfino, M. R.; *Curr. Pharm. Anal.* **2014**, *10*, 38.
12. Du, H.; Chen, X.; *J. Braz. Chem. Soc.* **2009**, *20*, 1268.
13. Orlandinia, S.; Gottib, R.; Furlanetto, S.; *J. Pharm. Biomed. Anal.* **2014**, *87*, 209.
14. Tavares, M. F. M.; *Quím. Nova* **1997**, *20*, 493.
15. Nishi, H.; Terabe, S.; *J. Chromatogr. A* **1996**, *735*, 3.
16. http://portal.anvisa.gov.br/wps/wcm/connect/4983b0004745975da005f43fbc4c6735/RE_899_2003_Determina+a+publica%C3%A7%C3%A3o+do+Guia+para+valida%C3%A7%C3%A3o+de+m%C3%A9todos+anal%C3%ADticos+e+bioanal%C3%ADticos.pdf?MOD=AJPERES accessed in June 2014.
17. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf accessed in June 2014.
18. César, I. D. C.; Pianetti, G. A.; *J. Pharm. Sci.* **2009**, *45*, 235.

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