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Original Paper

Validation of a capillary zone electrophoresis method for the comparative determination of etoricoxib in pharmaceutical formulations

A CZE method was developed and validated for the analysis of etoricoxib in pharmaceutical dosage forms, using prilocaine as an internal standard. The CZE method was carried out on a fused-silica capillary (50 μm id, effective length 40 cm). The BGE consisted of 25 mM tris-phosphate solution at pH 2.5. The capillary temperature was maintained at 35°C, the applied voltage was 25 kV, the injection was performed using the pressure mode at 50 mbar for 5 s, with detection at 234 nm using a photodiode array detector. The method was linear in the range of 2–150 μg/mL (r^2 = 0.9999). The specificity and stability-indicating capability were proven through the degradation studies and showing also that there was no interference of the excipients of the formulation. The accuracy was 99.49% with RSD of 0.66%. The limits of quantitation and detection were 2 and 0.58 μg/mL, respectively. Moreover, method validation demonstrated acceptable results for the precision, sensitivity, and robustness. The proposed method was successfully applied for the quantitative analysis of etoricoxib pharmaceutical formulations, and the results compared to the HPLC and LC-MS/MS methods, showing nonsignificant difference (p >0.05).

Keywords: Capillary zone electrophoresis / Etoricoxib / Liquid chromatography / Pharmaceutical analysis / Validation

Received: June 20, 2007; revised: August 16, 2007; accepted: August 17, 2007

DOI 10.1002/jssc.200700272

1 Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used agents in medicine, especially the group of coxibs that are selective cyclooxygenase-2 (COX-2) inhibitors. Etoricoxib (5-chloro-6′-methyl-3-[4-(methylsulfonyl) phenyl]-2,3(-bipyridine) (Fig. 1) represents a second-generation of COX-2 inhibitors that has been developed for the treatment of many inflammatory diseases such as rheumatoid arthritis, osteoarthritis, pain relief, and acute gout, causing fewer gastrointestinal complications than conventional NSAIDs [1–3].

A stability-indicating RP-HPLC was validated for both the impurity and quantitative analysis of etoricoxib using AQ-ODS column and gradient elution with detection at 220 nm [4]. The isocratic RP-HPLC was also validated for the determination of etoricoxib in pharmaceutical dosage forms using a C_{18} column and the results compared to the LC-MS/MS showing a significant correlation [5].

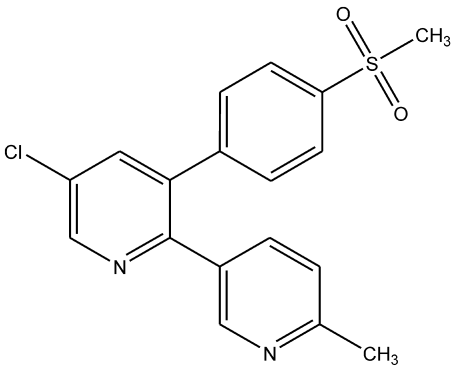

Figure 1. Chemical structure of etoricoxib.

An HPLC method was validated for the quantitation of etoricoxib in human plasma with a run time of 10 min [6]. An analytical HPLC method with photochemical cyclization and fluorescence detection was developed for the quantitation of etoricoxib in human plasma and urine, using a structural analog as the internal standard (IS) [7, 8]. The LC-MS/MS with positive ESI was validated for the

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Abbreviations: IS, internal standard; **PDA,** photodiode array

determination of etoricoxib in human plasma and pharmaceutical formulations [9]. The LC-MS/MS method with atmospheric pressure chemical ionization (APCI) and ESI were validated for the determination of etoricoxib in human plasma [10, 11].

CE has emerged as a powerful analytical technique for the pharmaceutical analysis allowing the determination of the active pharmaceutical ingredient, impurities, and chiral analysis, with some advantages related to the existing methodologies [12–15]. But, at the moment, there is no published CZE method for the determination of etoricoxib.

The aim of the present study was to develop and validate a stability-indicating CZE method for the determination of etoricoxib in solid pharmaceutical dosage forms, comparing the results with the HPLC and LC-MS/MS methods, and contributing to establish new alternatives with advantages for the quality control of the pharmaceutical formulations.

2 Experimental

2.1 Chemicals and reagents

Etoricoxib reference substance was generously supplied by Merck Research Laboratories (Rahway, USA) and prilocaine hydrochloride reference substance (IS) was purchased from United States Pharmacopoeia (Rockville, MD, USA). A total of six batches of Arcoxia® (Merck Sharp & Dohme, Haar, Germany) tablets, containing 60, 90, or 120 mg of etoricoxib were obtained from commercial sources within their shelf life period and were identified by Arabic numbers from 1 to 6. Analytical grade Tris-phosphate was purchased from Sigma (St. Louis, MO, USA), HPLC-grade ACN was from Tedia (Fairfield, OH, USA). All chemicals used were of pharmaceutical or special analytical grade. For all the analyses, ultrapure water was purified using an Elix 3 coupled to a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA). All solutions were degassed by ultrasonication (Tecnal, São Paulo, Brazil) and filtered through a 0.22 μm Millex filter (Millipore).

2.2 Apparatus

CE experiments were performed on an Agilent ^{3D}CE (Agilent Technologies, Waldbronn, Germany) apparatus consisting of autosampler, photodiode array (PDA) detector, temperature controlling system (4–60°C), and power supply able to deliver up to 30 kV. A CE ChemStation software was used for instrument control, data acquisition, and data analysis. The pH of the solutions was measured by a pH-meter (Thermo Orion Model 420 A, Beverly, MA, USA).

The HPLC method was carried out on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with an SCL-10A$_{VP}$ system controller, LC-10 AD$_{VP}$ pump, DGU-14A degasser, SIL-10AD$_{VP}$ autosampler, and a SPD-M10A$_{VP}$ PDA detector. The peak areas were integrated automatically by computer using a Shimadzu Class VP V 6.14 software program.

The LC-MS/MS method was performed on a Shimadzu HPLC system (Shimadzu) equipped with an SCL-10A$_{VP}$ system controller, LC-10 AD$_{VP}$ pump, DGU-14A degasser, and a CTO-10 AD$_{VP}$ column oven. A triathlon autosampler (Spark, Emmen, Holland) was used. The triple quadrupole mass spectrometer (Micromass, Manchester, UK), model Quattro LC, equipped with an electrospray source using a crossflow counter electrode run in a positive mode, was set up in a multiple reaction monitoring (MRM) mode, monitoring the transitions 359.3 > 280.0 and 332.0 > 95.0, for etoricoxib and piroxicam (IS), respectively. The peak areas were integrated automatically by computer using a Masslynx (v 3.5) software program.

2.3 Electrophoretic procedure

Optimized running electrolyte solution was 25 mM tris-phosphate in ultrapure water, adjusted to pH 2.5 with 85% phosphoric acid, daily prepared and filtered. At the beginning of each working day, the capillary was conditioned by rinsing with 1 M sodium hydroxide for 10 min, followed by water for 10 min, and then with running electrolyte for 15 min. The electrolyte solution was prepared and filtered daily. To achieve high migration time reproducibility between injections, the capillary was conditioned with running buffer for 5 min. All experiments were carried out on a fused-silica capillary with 50 μm id and 48.5 cm of total length (effective length 40 cm, HP part No. G1600-60211), thermostatized at 35°C, and detected at 234 nm using a PDA detector. Samples were injected using the pressure mode for 5 s at 50 mbar and a constant voltage of 25 kV (current about 33 μA) was applied during the analysis. As electrolysis can change the EOF and affect migration time, efficiency, and selectivity, the running electrolyte was replaced with a fresh electrolyte after each three injections. BGE consisted of a 25 mM tris-phosphate solution at pH 2.5.

2.4 HPLC and LC-MS/MS procedures

The validated HPLC and LC-MS/MS methods applied for the analysis of etoricoxib in pharmaceutical dosage forms are described elsewhere [5]. Briefly, for the HPLC method, the elution was carried out on a RP Phenomenex (Torrance, USA) Synergi fusion C_{18} column (150 mm × 4.6 mm id, with a particle size of 4 μm and pore size of 80 Å). A security guard holder (4.0 mm × 3.0 mm id) was used to protect the analytical

column. The Shimadzu HPLC system was operated isocratically at ambient controlled temperature (25°C) using a mobile phase of phosphoric acid 0.01 M, pH 3.0/ACN (62:38, v/v), run at a flow-rate of 1.0 mL/min, and using PDA detection at 234 nm. The injection volume was 10 μL of a solution containing 100 μg/mL for both reference substance and samples.

For the LC-MS/MS method, the experiments were carried out on a RP Phenomenex Luna C_{18} column (50 mm × 3.0 mm id, with a particle size of 3 μm and pore size of 100 Å). A security guard holder (4.0 mm × 3.0 mm id) was used to protect the analytical column. The HPLC system was operated isocratically at controlled temperature (40°C) using a mobile phase of ACN/water (95:5)/0.1% acetic acid (90:10, v/v), run at a flow rate of 0.4 mL/min. The electrospray capillary potential was set to 3.2 kV. The cone voltage was 62 and 32 V and the collision energy was 30 and 20 eV, respectively, for etoricoxib and IS. For the pharmaceutical products assay seven calibration points were prepared and diluted with ACN/water (1:1, v/v) to obtain the concentration range from 1 to 5000 ng/mL. The calibration reference substance and the pharmaceutical samples were spiked with 25 μL of IS solution (500 ng/mL in ACN). The injection volume was 20 μL for both reference substance and samples.

2.5 Preparation of reference substance solutions

The stock solutions of etoricoxib and prilocaine were prepared by weighing 10 mg of each reference substance, transferred to individual 10 mL volumetric flasks and diluted to volume with ACN, obtaining a concentration of 1 mg/mL. The stock solutions were stored at 2 – 8°C protected from light and daily diluted to an appropriate concentration with BGE.

2.6 Preparation of sample solutions

To prepare the sample stock solutions, tablets containing 60, 90, or 120 mg of etoricoxib were accurately weighed and crushed to a fine powder. An appropriate amount was transferred into an individual 50 mL volumetric flask, diluted to volume with ACN, and sonicated for 15 min, obtaining the final concentration of 1 mg/mL of the active pharmaceutical ingredient. This solution was stored at 2 – 8°C protected from light and daily diluted to an appropriate concentration with BGE.

2.7 Validation of the method

The method was validated using samples of pharmaceutical formulations by the determination of the following parameters: specificity, linearity, range, precision, accuracy, LOD, LOQ, robustness, and system suitability test, following the ICH guidelines [16, 17]. Prilocaine was used as IS to compensate injection errors and minor fluctuations of the migration time, improving the reproducibility of the CZE method.

2.7.1 Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities [17]. The stability-indicating capability of the method was determined by subjecting a reference substance solution (100 μg/mL) to accelerated degradation by acidic, basic, neutral, oxidative, and photolytic conditions to evaluate the interference in the quantitation of etoricoxib. After the procedures, the samples were diluted in the electrolyte solution to a final concentration of 50 μg/mL. A sample solution prepared in 0.1 M hydrochloric acid was used for the acidic hydrolysis, and a sample solution in 0.1 M sodium hydroxide for the basic hydrolysis evaluation. Both solutions were refluxed at 100°C for 4 h, cooled, and neutralized with acid or base, as necessary. For the study in neutral condition, the samples were diluted in water and heated at 80°C for 3 h. The oxidative degradation was induced by storing the samples solutions in 5% hydrogen peroxide, at an ambient temperature for 24 h, protected from light. Photodegradation was induced by exposing the samples to 200 W · h/m² of near UV light for 24 h. The interference of the excipients of the pharmaceutical formulation was determined by the injection of a sample containing only placebo (in-house mixture of all the tablet excipients) and a sample containing placebo added with etoricoxib at a concentration of 50 μg/mL. Then, the specificity of the method was established by determining the peak purity of etoricoxib in the degraded samples using a PDA detector.

2.7.2 Linearity and range

Linearity was determined by constructing three calibration curves each one with seven calibration points of etoricoxib, including the LOQ, in the range of 2 – 150 μg/mL prepared in BGE. Three replicate injections of each reference substance solution were made to verify the repeatability of the detector response. The peak area ratio of etoricoxib to IS against the respective reference substance concentrations was used for plotting the graph, and the linearity was evaluated by the least-square regression analysis.

2.7.3 Precision and accuracy

The precision of the method was determined by repeatability and intermediate precision. Repeatability was examined by eight evaluations of the same concentration sample of etoricoxib, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the anal-

ysis on three different days (interdays) and also by other analysts performing the analysis in the same laboratory (between-analysts). The accuracy was evaluated by applying the proposed method to the analysis of the in-house mixture of the tablet excipients with known amounts of each drug, to obtain solutions at concentrations of 40, 50, and 60 μg/mL, equivalent to 80, 100, and 120%, of the analytical concentrations, respectively. The accuracy was calculated as the percentage of the drug recovered from the formulation matrix.

2.7.4 LOQ and LOD

The LOQ and the LOD were calculated from the slope and the SD of the intercept of the mean of three calibration curves, determined by a linear regression model, as defined by ICH. The LOQ was also evaluated in an experimental assay.

2.7.5 Robustness

The robustness was determined by analyzing the same samples under a variety of conditions of the method parameters, such as: electrolyte solution pH and concentration, capillary temperature, and voltage applied. To assess the stability of etoricoxib, the samples were maintained at 4–8°C for 48 h and also placed into the autosampler at room temperature for 48 h.

2.8 Analysis of pharmaceutical tablet dosage forms

For the quantitation of etoricoxib in the pharmaceutical dosage forms, 20 tablets of each sample containing 60, 90, or 120 mg of etoricoxib were separated, accurately weighed, and crushed to a fine powder. An appropriate amount was transferred into an individual 50 mL volumetric flask, diluted to volume with ACN, and sonicated for 15 min, obtaining final concentration of 1 mg/mL (stock solution). For the analysis, the stock solutions were daily diluted to appropriate concentrations with BGE, injected and the amount of the drug *per* tablet calculated against the reference substance.

3 Results and discussion

3.1 Optimization of the electrophoretic conditions

To develop the CZE method, the influences of different parameters on migration time, peak symmetry, and electric current were optimized. The optimum tris-phosphate electrolyte solution pH 2.5, which determines the degree of ionization in the analyte and its electrophoretic mobility, was investigated in the range of 2.0–4.0. The migration time of etoricoxib increased with the increase of the pH, and the pH 2.5 was selected due to the better peak symmetry (about 0.95).

The tris-phosphate electrolyte solution in the concentration range of 5–35 mM was evaluated by showing a significant effect on the separation performance through its influence on the EOF and the current produced in the capillary. The 25 mM tris-phosphate electrolyte solution was selected due to its low current and no significant increase in the migration time. The effects of the organic modifiers ACN or methanol, in the concentration range of 5–20%, were also evaluated, but no improvement in the electrophoretic conditions was achieved.

The temperature effect on the separation was investigated in the range of 20–45°C. An increase in the capillary temperature resulted in a decrease of migration time and an increase in the current. The temperature was chosen as 35°C due to the short run time, peak symmetry, and acceptable current. The effect of the voltage applied on the separation was studied through changes from 10 to 30 kV, showing that the potential of 25 kV yielded the short analysis time with acceptable current (about 33 μA).

3.2 Method validation

The stability-indicating CZE method was validated for the analysis of etoricoxib in pharmaceutical dosage forms, with the migration time of approximately 4.9 min, as shown in the typical electropherogram in Fig. 2.

The stability-indicating capability of the analytical method evaluated under photolytic and neutral conditions resulted in a nonsignificant decrease in the area. As indicated in Fig. 3, under the basic condition the etoricoxib content exhibited decrease in the area without any additional peak (Fig. 3B), indicating that the degradation products do not migrate or were not detected by UV. The oxidative condition exhibited significant decrease and three additional peaks were detected at 4.70, 5.47, and 5.67 min (Fig. 3C). Under the acidic condition, a decrease in the area was observed, with one additional peak at 3.95 min (Fig. 3D). Specificity of the method towards the drug was established through determination of peak purity of the analyte and the IS in the working reference substance solution. Peak purity was obtained by overlay of the spectra captured at the apex, upslope, and downslope using a PDA detector. No interference from formulation excipients was found, showing that the peak was free from any coeluting peak, thus demonstrating that the proposed method is specific for the analysis of etoricoxib.

The calibration curves constructed for etoricoxib were found to be linear in the 2–150 μg/mL range. The value of the determination coefficient calculated ($r^2 = 0.9999$, $y = 0.0884 \pm 0.0014x \pm 0.0172 \pm 0.0172$, where x is the concentration and y is the peak area ratio of etoricoxib to IS)

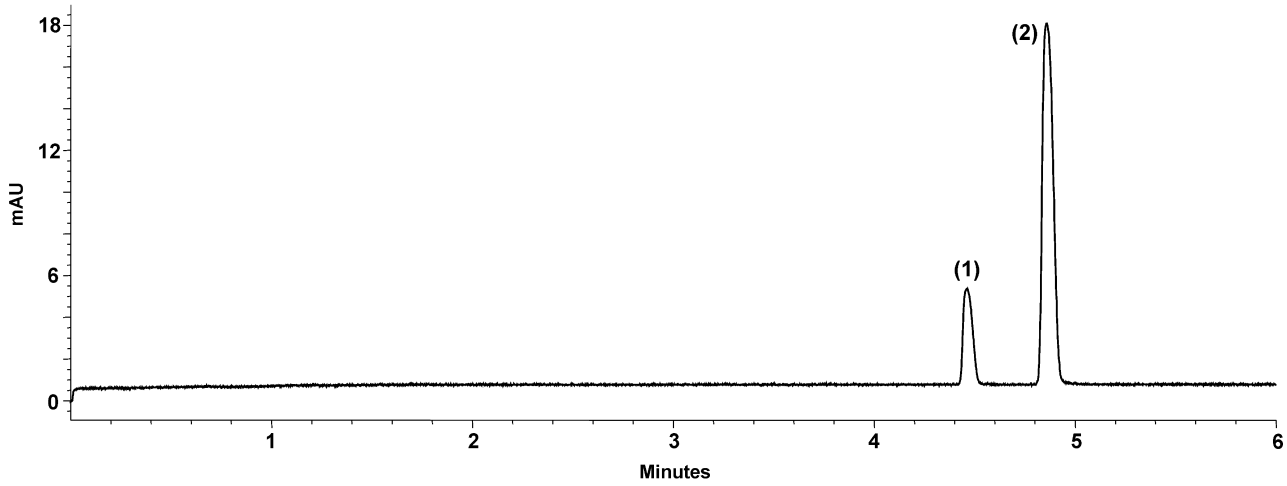

Figure 2. Representative electropherogram of (1) prilocaine and (2) etoricoxib at a concentration of 50 μg/mL. Operating conditions: tris-phosphate electrolyte solution (25 mM, pH 2.5), hydrodynamic injection (5 s at 50 mbar), 25 kV, 35°C, and 234 nm.

Table 1. Interday precision data of CZE for etoricoxib in samples of pharmaceutical formulation

Sample	Day	Recovery[a] (%)	Mean[b] (%)	RSD[c] (%)
	1	99.44		
1	2	99.66	99.30	0.45
	3	98.80		
2	1	99.86	99.41	0.40
	2	99.20		
	3	99.16		

[a] Mean of three replicates.
[b] Mean of three days.
[c] RSD, Relative standard deviation.

Table 2. Between-analysts precision data of CZE for etoricoxib in samples of pharmaceutical formulation

Sample	Analyst	Recovery[a] (%)	Mean[b] (%)	RSD[c] (%)
	A	100.02		
1	B	99.89	100.01	0.11
	C	100.11		
2	A	100.19	100.00	0.17
	B	99.95		
	C	99.87		

[a] Mean of three replicates.
[b] Mean of three analysts.
[c] RSD, Relative standard deviation.

indicated the linearity of the calibration curve for the method.

The precision evaluated as the repeatability of the method was studied by calculating the RSD% of the migration time and peak area ratio of eight analysis of the concentration of 50 μg/mL, performed on the same day, under the same experimental conditions. The RSD values obtained were 0.63 and 0.60% for the migration time and peak area ratio, respectively.

The intermediate precision was assessed by analyzing two samples of the pharmaceutical formulation on three different days (interday, Table 1); the RSD values obtained were 0.45 and 0.40%. Between-analysts precision was determined by calculating the RSD for the analysis of two samples of the pharmaceutical formulation by three analysts; the values were found to be 0.11 and 0.17% (Table 2).

The accuracy was assessed from three replicate determinations of three different solutions containing 40, 50, or 60 μg/mL, corresponding to 80, 100, or 120%, of the analytical concentrations, respectively. The absolute means obtained with a mean value of 99.49% and RSD of 0.66% are shown in Table 3, demonstrating that the method is accurate within the desired range.

Table 3. Accuracy of CZE for etoricoxib in samples of pharmaceutical formulation

Nominal concentration (μg/mL)	Mean concentration found[a] (μg/mL)	Accuracy (%)	Mean ± RSD[b] %
40	39.57	98.93	
50	50.10	100.21	99.49 ± 0.66
60	59.60	99.34	

[a] Mean of three replicates.
[b] RSD, Relative standard deviation.

For the calculation of the LOD and LOQ, the calibration equations for etoricoxib were generated by using the mean values of the three independent calibration curves and the values obtained were 0.58 and 1.94 μg/mL, respectively. The LOQ evaluated experimentally, with the precision lower than 5% and accuracy within ±5%, was found to be 2.0 μg/mL.

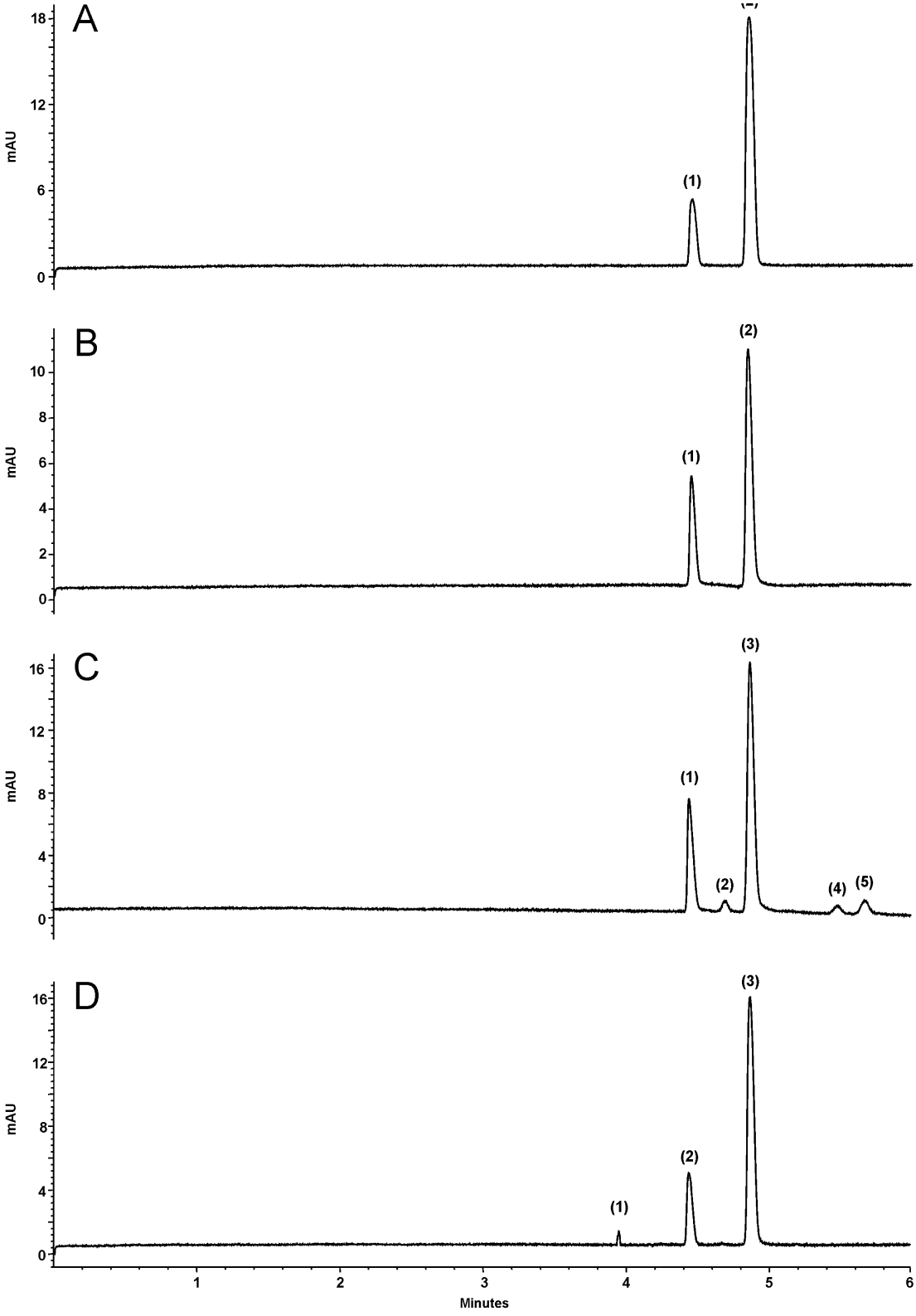

Figure 3. CZE electropherograms of etoricoxib. (A) Etoricoxib reference substance solution (50 μg/mL): peak 1, prilocaine; peak 2, etoricoxib. (B) After basic hydrolysis: peak 1, prilocaine; peak 2, etoricoxib. (C) After oxidation: peak 1, prilocaine; peak 3, etoricoxib; peaks 2, 4, and 5, photodegraded forms. (D) After acidic hydrolysis: peak 1, degraded form; peak 2, prilocaine; peak 3, etoricoxib.

The results and the experimental range of the variables evaluated in the robustness assessment are given in Table 4, together with the optimized conditions. There were no significant changes in the CZE pattern when the modifications were made in the experimental conditions, thus showing the method to be robust. The solutions were also found to be stable during the study period.

The system suitability test was also carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed, using five replicate injections of a reference substance solution containing 50 μg/mL of etoricoxib. The results given in Table 5 show that the parameters evaluated are within the suitable range.

Table 4. CZE conditions and range investigated during robustness testing

Variable	Range investigated	Etoricoxib[a] (%)	Optimized condition
Electrolyte solution pH	2.2	99.06	2.5
	2.4	98.87	
	2.5	99.92	
	2.6	99.62	
	2.8	99.53	
Electrolyte solution concentration (mM)	23	99.91	25
	24	101.62	
	25	99.98	
	26	101.36	
	27	100.62	
Temperature (°C)	33	100.95	35
	35	99.28	
	37	101.97	
Voltage (kV)	23	101.25	25
	25	99.59	
	27	100.68	
Solution stability	Autosampler/48 h	98.60	–
	4 – 8°C/48 h	99.07	

[a] Mean of three replicates.

Table 5. Results of the system suitability test

Parameter	Etoricoxib[a])			
	Minimum	Maximum	RSD[b] (%)	Status
Peak symmetry	0.956	0.982	1.20	Passed
Theoretical plates	32 513	33 087	0.74	Passed
Migration time	4.87	4.95	0.59	Passed
Area	61.99	64.12	1.33	Passed

[a] Mean of five replicates.
[b] RSD, Relative standard deviation.

3.3 Method application

The CZE method validated in this paper was applied for the determination of etoricoxib in tablet dosage forms as shown in Table 6, together with the results obtained by the HPLC and LC-MS/MS methods, respectively. The experimental values obtained from the three methods were compared statistically by ANOVA showing mean values 1.17 and 0.35% lower for the CZE compared to HPLC and LC-MS/MS methods, respectively, with nonsignificant difference ($p > 0.05$). The proposed method can be useful for the determination of etoricoxib without prior separation of the excipients of the formulation, with advantages of small sample volumes, low consumption of reagents and organic solvents, and short analysis time. The application for the analysis of spiked human plasma samples was also evaluated showing lower sensitivity due to the low injection volume and the short optical path-length, compared to the existing methods.

4 Concluding remarks

The results of the validation studies show that the CZE method is economic, specific, stability-indicating, sensitive, accurate, and possesses significant linearity and precision characteristics without any interference from the excipients. Therefore, the proposed method was successfully applied as an alternative for the quantitative anal-

Table 6. Comparison between CZE, HPLC, and LC-MS/MS methods in the assay of pharmaceutical formulations

Theoretical amount		Experimental amount								
		CZE[a]			HPLC[a]			LC-MS/MS[a]		
Sample	mg *per* tablet	mg	%	RSD[b] (%)	mg	%	RSD[b] (%)	mg	%	RSD[b] (%)
1	60	57.09	95.15	0.92	58.03	96.72	1.62	58.30	97.17	1.60
2	60	57.73	96.22	1.57	58.19	96.98	1.18	57.56	95.93	0.51
3	90	91.04	101.16	0.42	91.03	101.14	0.90	90.98	101.09	1.94
4	90	89.51	99.46	0.33	90.69	100.77	0.37	89.96	99.96	1.45
5	120	114.44	95.37	0.08	117.08	97.57	1.07	114.83	95.69	1.35
6	120	116.50	97.08	0.47	117.71	98.09	0.87	115.99	96.66	1.58

[a] Mean of three replicates.
[b] RSD, Relative standard deviation.

ysis of etoricoxib in pharmaceutical dosage forms, representing an improvement for the quality control, and contributing also to assure the therapeutic efficacy of the drug.

The authors wish to thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, project no. 305148/2005-2) for the financial support.

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