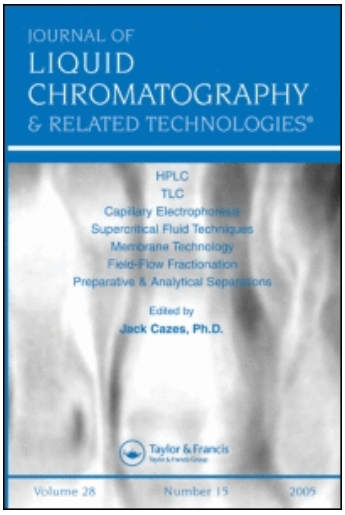

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Abstract: A micellar electrokinetic capillary chromatography (MEKC) method for the analysis of nimesulide and valdecoxib, using celecoxib as internal standard, has been developed and validated. The MEKC method was carried out on a fused silica capillary (50 μm I.D., effective length 56 cm). The background electrolyte consisted of 35 mM borate buffer and 35 mM of anionic detergent SDS (pH 9.75)/acetonitrile (95:5, V/V). The capillary temperature was maintained at 35°C, the applied voltage was 30 kV, and the injection was performed using the pressure mode at 50 mbar for 5 s, with detection at 234 nm using a photodiode array detector. Method validation investigated parameters such as the linearity ($r^2 = 0.9999$), range, precision, accuracy, robustness, and specificity, giving results within the acceptable range. The detection limit calculated for nimesulide and valdecoxib was 0.25 and 0.86 μg mL^{-1}, respectively, and the quantitation limit evaluated experimentally was 2 μg mL^{-1} for both the compounds. The proposed method was successfully applied for the quality control analysis of pharmaceutical products and the results compared to the liquid chromatography method, showing non-significant difference ($P > 0.05$).

Keywords: Nimesulide, Valdecoxib, Micellar electrokinetic capillary chromatography, Validation, Pharmaceutical analysis

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INTRODUCTION

Nimesulide [methanesulfonamide, *N*-(4-nitro-2-phenoxyphenyl)] and valdecoxib [4-(5-methyl-3-phenylisoxazol-4-yl) benzenesulfonamide] (Figure 1) are non-steroidal anti-inflammatory drugs with selective inhibitor effects on the cyclooxygenase-2 (COX-2) enzyme, induced at sites of pain and inflammation. These drugs are clinically useful for the therapeutic management of various inflammatory conditions.[1–3] Due to the recent findings related to the cardiovascular risk or serious skin reactions, valdecoxib is under investigation to understand the mechanistic basis and to evaluate the safe dosage level, also, as a potential new candidate for prevention and treatment of cancer.[4,5]

An isocratic reversed-phase liquid chromatography (RP-LC) method was developed and applied for the determination of nimesulide in pharmaceutical dosage forms and stability studies, using a C_{18} column and UV detection.[6] The isocratic RP-LC method was also validated to monitor the degradation products of nimesulide exposed to the 254 nm light, detecting the presence of 2-phenoxy-4-nitroaniline. The methanesulphonic acid was evaluated by a thin layer chromatography procedure, applied also for the analysis of nimesulide in bulk and pharmaceutical formulations, with the quantitation limit of 100 ng.[7,8] A RP-LC method with liquid-liquid extraction was developed for the determination of nimesulide in human plasma, using a structurally similar analogue as internal standard, and applied for a pilot pharmacokinetic

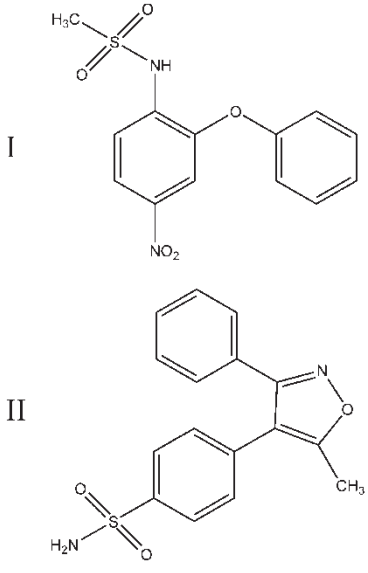

Figure 1. Chemical structures of nimesulide (I) and valdecoxib (II).

study in rats.[9] A high throughput liquid chromatography method was validated with a short monolithic column and used for accelerated and long term dissolution stability studies of the nimesulide containing tablets, showing acceptable parameters and selectivity against potential impurities of the active ingredient.[10]

An isocratic RP-LC procedure using a C_{18} analytical column and UV detection was developed and validated for the dissolution rate studies and quantitative determination of valdecoxib in solid dosage forms.[11] A RP-LC method was validated for the determination of valdecoxib in human plasma using liquid-liquid extraction, with a quantitation limit of 10 ng/mL.[12] RP-LC methods with UV detection were described for the pharmacokinetics studies of valdecoxib in human volunteers[13] and for the separation of valdecoxib from its impurities in film coated tablets.[14] An automated solid phase extraction liquid chromatography-tandem mass spectrometry (LC-MS/MS) method using electrospray ionization in negative ion mode was developed and validated for the determination of valdecoxib and its metabolites in human urine and plasma.[15,16]

An LC-MS/MS method, performed in the positive ion mode, was described for the determination of both valdecoxib and etoricoxib in human plasma and applied to evaluate the pharmacokinetics profiles in volunteers.[17] Simultaneous determinations of COX-2 inhibitors including valdecoxib and nimesulide, were also performed in pharmaceuticals and biological fluids by isocratic and gradient RP-LC methods.[18,19] The RP-LC methods and the columns tested for the analysis of nimesulide, and the mass spectrometer method, used for valdecoxib, were recently published in a review of the developments in analytical methodologies for the determination of COX-2 inhibitors in bulk drugs, pharmaceuticals, and biological matrices.[20]

Capillary electrophoresis (CE) has emerged as a powerful technique for pharmaceutical analysis allowing the determination of the drug, drug related impurities, residues, and chiral analysis.[21–24] The aim of the present study was to develop and validate an efficient, reliable, accurate, and sensitive micellar electrokinetic chromatography (MEKC) method for the simultaneous determination of nimesulide and valdecoxib in solid pharmaceutical dosage forms, comparing the results to the LC method, and contributing to establishing new alternatives for the quality control of the pharmaceutical formulations.

EXPERIMENTAL

Chemicals and Reagents

Nimesulide reference standard was purchased from European Pharmacopoeia, and Valdecoxib reference standard was generously supplied by Pfizer Laboratories (Kalamazoo, USA). Celecoxib (internal standard, IS) was obtained from

Sequoia Research products (Oxford, United Kingdom). A total of five batches of Valdecoxib tablets containing 10, 20, or 40 mg of the active substance, and nimesulide tablets containing 100 mg of the active pharmaceutical ingredients were obtained from commercial sources and used within their shelf life period. LC-grade acetonitrile and methanol were purchased from Tedia (Fairfield, USA). Ultrapure sodium dodecyl sulphate (SDS electrophoresis) and analytical grade boric acid were acquired from Bio-Rad Labs (Hercules, CA, USA) and Merck (Darmstadt, Germany), respectively. For all the analyses, ultrapure water (Millipore, Bedford, MA, USA) filtered through a 0.22 μm membrane filter was used. All solutions were degassed by ultrasonication (Tecnal, São Paulo, Brazil) and filtered through a 0.22 μm Millex filter (Millipore, Bedford, MA, USA).

Apparatus

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

Electrophoretic Procedure

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 equilibrated with the running buffer for 5 min. All experiments were carried out in a fused silica capillary with 50 μm I.D. and 64.5 cm of total length (effective length 56 cm, HP part N° G1600-61211), thermostatted at 35°C, and detection 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 30 kV (current about 36 μA) was applied during the analysis. As electrolysis can change the electroosmotic flow (EOF) and affect migration time, efficiency, and selectivity, the running electrolyte was replaced with a fresh electrolyte after each three injections. Background electrolyte (BGE) consisted of a mixture of 35 mM

aqueous borate buffer and 35 mM anionic detergent SDS (pH 9.75) with acetonitrile (95:5, V/V).

Chromatographic Procedure

The validated LC method applied for the analysis of valdecoxib in pharmaceutical dosage forms is described elsewhere.[17] Briefly, the elution was carried out on a reversed-phase Phenomenex (Torrance, USA) Synergi fusion C_{18} column (150 mm × 4.6 mm I.D., with a particle size of 4 μm and pore size of 80 Å). A security guard holder (4.0 mm × 3.0 mm I.D.) was used to protect the analytical column. The Shimadzu HPLC system was operated isocratically at 30°C using a mobile phase of water (pH 7.0)/acetonitrile (52:48, V/V), run at a flow rate of 1.0 mL min^{-1}, and using PDA detection at 210 nm. The injection volume was 10 μL of a solution containing 50 μg mL^{-1} for both standards and samples. Moreover, the analysis of nimesulide was carried out on a reversed-phase Phenomenex Synergi fusion C_{18} column (150 mm × 4.6 mm I.D., with a particle size of 4 μm and pore size of 80 Å). A security guard holder (4.0 mm × 3.0 mm I.D.) was used to protect the analytical column. The Shimadzu HPLC system was operated isocratically using a mobile phase of potassium phosphate buffer (0.05 M, pH 4.4)/methanol (40:60, V/V), run at a flow rate of 1.0 mL min^{-1}, and using PDA detection at 230 nm. The injection volume was 10 μL of a solution containing 50 μg mL^{-1} for both standards and samples.

Preparation of Reference Solutions

The stock solution of valdecoxib was prepared by weighing 10 mg of the reference substance, transferred to individual 10 mL volumetric flasks, and diluted to volume with methanol, obtaining a concentration of 1 mg mL^{-1}. Equally, nimesulide and celecoxib stock solutions were also made at a final concentration of 1 mg mL^{-1} using acetonitrile. The stock solutions were stored at 4–8°C protected from light and daily diluted to an appropriate concentration with BGE.

Preparation of Sample Solutions

To prepare the sample stock solutions, tablets containing 10, 20, and 40 mg of valdecoxib and 100 mg of nimesulide, respectively, were accurately weighed and crushed to a fine powder. An appropriated amount was transferred into individual 50 mL volumetric flasks, diluted to volume with methanol for valdecoxib and with acetonitrile for nimesulide, and sonicated for 15 min, obtaining the final concentration of 1 mg mL^{-1} of the active pharmaceutical

ingredient. The solutions were stored at 4–8°C protected from light, and daily diluted to an appropriate concentration with BGE.

Validation of the Method

The method was validated using samples of pharmaceutical formulations with the label claim of 100 mg of nimesulide and 10 mg of valdecoxib by the determination of the following parameters: specificity, linearity, range, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness, following the ICH guidelines.[25] Celecoxib was used as internal standard (IS) to compensate injection errors and minor fluctuations of the migration time, improving the reproducibility of the MEKC method.

Specificity

The interference of the excipients of the pharmaceutical formulations was determined by the injection of a sample containing only placebo (in-house mixture of all the tablets excipients) and a sample containing placebo added with nimesulide and valdecoxib at a concentration of 50 $\mu g\ mL^{-1}$. Then, the specificity of the method was established by determining the peak purity of the samples using a PDA detector.

Linearity and Range

Linearity was determined by constructing three calibration curves, each one with seven standard concentrations of nimesulide and valdecoxib, in the range of 5–150 $\mu g\ mL^{-1}$ prepared in BGE. Three replicate injections of each standard solution were made to verify the repeatability of the detector response. The peak area ratio of each analyte to IS against the respective standard concentrations was used for plotting the graph, and the linearity was evaluated by least square regression analysis.

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 nimesulide and valdecoxib, on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on three different days (inter-days) and also by other analysts performing the analysis in the same laboratory (between-analysts). The accuracy was evaluated applying the proposed method to the analysis of the in-house mixture of the respective tablet excipients with known amounts of the drug, to obtain solutions at concentrations of 40, 50, and 60 $\mu g\ mL^{-1}$, equivalent to 80, 100, and 120%,

respectively. The accuracy was calculated as the percentage of the drug recovered from the formulation matrix.

Limit of Quantitation and Limit of Detection

The limit of quantitation (LOQ) and the limit of detection (LOD) were calculated from the slope and the standard deviation 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.

Robustness

The robustness was determined by analyzing the same samples under a variety of conditions of the method parameters, such as: buffer pH and concentration, SDS concentration, and percentage of acetonitrile in the BGE. To assess the stability of nimesulide and valdecoxib, the samples were tested maintained at 4–8°C for 48 h and also placed into the autosampler, at room temperature, for 48 h.

Analysis of Pharmaceutical Tablet Dosage Forms

For the quantitation of the pharmaceutical dosage forms, twenty tablets of the respective sample containing 100 mg of nimesulide, and 10, 20, or 40 mg of valdecoxib were separated, accurately weighed, and crushed to a fine powder. An appropriate amount of each tablet dosage form was transferred into an individual 50 mL volumetric flask, diluted to volume with acetonitrile, and sonicated for 15 min, obtaining final concentrations of 1 mg mL^{-1} (stock solution). For the analysis, the stock solutions were daily diluted to appropriate concentrations with BGE, injected, and the amount of the drugs per tablet calculated against the reference standard.

RESULTS AND DISCUSSION

Optimization of the Electrophoretic Conditions

To develop the CE method, the influence of different parameters on migration time, peak symmetry, and electric current was optimized. The optimum borate buffer pH, which determines the degree of ionization of the analyte and its electrophoretic mobility, was investigated in the range of 8.0–11.0. The migration time of the analytes increased with the increase of the pH and the pH of 9.75 was chosen due to the better peak symmetry (about 0.96).

The borate buffer in the concentration range of 10–50 mM was evaluated showing a significant effect on the separation performance through its influence on the EOF and the current produced in the capillary. The 35 mM

borate buffer was selected due to its low current and no significant increase on the migration time. Then, the MEKC was applied and the effect of anionic detergent SDS was assessed in the range of 15–45 mM, selecting the concentration of 35 mM due to the sharp peaks and acceptable current achieved. The effects of the organic modifiers acetonitrile or methanol, in the range of 5–20%, were also evaluated. The best result regarding to peak symmetry (about 0.96) was achieved with 5% of acetonitrile (V/V) in the BGE, without a significant increase on the migration time.

The temperature effect on the separation was investigated in the range of 20–45°C. An increase of the capillary temperature resulted in a decrease of migration time and increase of the current. The temperature of 35°C was chosen due to the short run time, peak shape, and acceptable current. The effect of the voltage applied on the separation was studied through changes from 15 to 30 kV, showing that the potential of 30 kV yielded the shortest analysis time with acceptable current (about 36 μA).

Method Validation

The MEKC method was validated for the analysis of nimesulide and valdecoxib in pharmaceutical dosage forms, with the migration times of 4.31 and 6.88 minutes, respectively, as shown in the typical electropherogram in Figure 2.

Specificity of the method towards the drug was established through determination of purity peak of the analytes and the IS in the working standard solution. Peak purity was obtained by overlay of the spectra captured at the apex, up slope, and down slope using a photodiode array detector. No interference from formulation excipients was found, showing that each peak was free

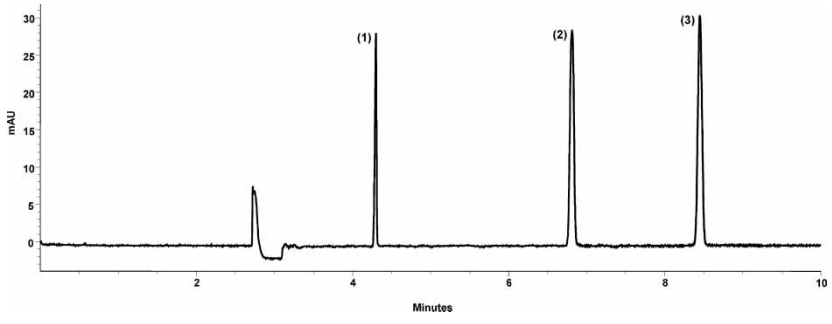

Figure 2. Representative electropherogram of (1) nimesulide, (2) valdecoxib, and (3) celecoxib at a concentration of 50 μg mL^{-1}. Operating conditions: 35 mM borate buffer and 35 mM SDS (pH 9.75)/acetonitrile (95:5, V/V), hydrodynamic injection (5 s at 50 mbar), 30 kV, 35°C, and 234 nm.

from any coeluting peak, thus demonstrating that the proposed method is specific for the analysis of nimesulide and valdecoxib.

The calibration curves constructed for nimesulide and valdecoxib were found to be linear in the 5–150 μg mL^{-1} range. The values of the determination coefficient calculated ($r^2 = 0.9999$, $y = 0.007567 \pm 0.000115x + 0.009033 \pm 0.000643$; and $r^2 = 0.9999$, $y = 0.0178 \pm 0.000058x + 0.0019 \pm 0.005122$ for nimesulide and valdecoxib, respectively, where, x is concentration and y is the peak area ratio of each analyte to IS) indicated the linearity of the calibration curves for the method.

The precision evaluated as the repeatability of the method was studied by calculating the relative standard deviation (RSD%) of the migration time and peak area ratio of eight analyses of the concentration of 50 μg mL^{-1} performed on the same day under the same experimental conditions. The RSD values obtained for the migration time and peak area ratio were 0.33 and 0.82%, respectively, for nimesulide and 0.51 and 0.30% for valdecoxib.

The intermediate precision was assessed by analyzing two samples of the pharmaceutical formulation on three different days (inter-day, Table 1); the RSD values obtained were 0.34 and 0.82% for nimesulide and 0.82 and 0.86% for valdecoxib, respectively. 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 0.22 and 0.20% for nimesulide and 0.38 and 0.03% for valdecoxib, respectively (Table 2).

The accuracy was assessed from three replicate determinations of three different solutions containing 40, 50, and 60 μg mL^{-1}, corresponding to 80,

Table 1. Inter-day precision data of MEKC for nimesulide and valdecoxib in samples of pharmaceutical formulation

Sample	Drug	Day	Recovery[a] (%)	Mean[b]	RSD[c] (%)
1	Nimesulide	1	99.43	99.82	0.34
		2	99.96		
		3	100.08		
	Valdecoxib	1	99.50	99.09	0.82
		2	99.61		
		3	98.15		
2	Nimesulide	1	99.40	100.19	0.82
		2	100.13		
		3	101.04		
	Valdecoxib	1	99.76	99.26	0.86
		2	99.75		
		3	98.27		

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

100, and 120%, respectively. The absolute means obtained for nimesulide were 101.65, 99.40, and 99.70%, respectively, with a mean value of 100.25% and RSD of 1.22%. The corresponding values obtained for valdecoxib were 99.36, 99.50, and 98.51%, respectively, as shown in Table 3, with a mean value of 99.12% and RSD of 0.54%, demonstrating that the method is accurate within the desired range.

For the calculation of the LOD and LOQ, the calibration equations for nimesulide and valdecoxib were generated by using the mean values of the three independent calibration curves, and the values obtained were 0.25 and 0.85 $\mu g\ mL^{-1}$ for nimesulide, and 0.86 and 2.88 $\mu g\ mL^{-1}$ for valdecoxib, respectively. The LOQ evaluated experimentally for each compound, with the precision lower than 5% and accuracy within $\pm 5\%$, was found to be 2 $\mu g\ mL^{-1}$ for both drugs.

The results and the experimental range of the variables evaluated in the robustness assessment are given in Table 4, together with the optimized values. There were no significant changes in the MEKC 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 standard solution containing 50 $\mu g\ mL^{-1}$ of nimesulide and valdecoxib. The results given in Table 5 show that the parameters are within the suitable range.

Table 2. Between-analysts precision data of MEKC for nimesulide and valdecoxib in samples of pharmaceutical formulation

Analyst	Drug	Sample	Recovery[a] (%)	Mean[b]	RSD[c] (%)
A	Nimesulide	1	98.49	98.70	0.22
		2	98.68		
		3	98.93		
	Valdecoxib	1	98.85	99.26	0.38
		2	99.35		
		3	99.59		
B	Nimesulide	1	99.30	99.07	0.20
		2	98.99		
		3	98.93		
	Valdecoxib	1	99.80	99.83	0.03
		2	99.87		
		3	99.83		

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

Table 3. Accuracy of MEKC for nimesulide and valdecoxib in samples of pharmaceutical formulation

Drug	Nominal concentration ($\mu g\ mL^{-1}$)	Mean concentration found[a] ($\mu g\ mL^{-1}$)	RSD[b] (%)	Accuracy (%)
Nimesulide	40	40.66	0.83	101.65
	50	49.70	0.74	99.40
	60	59.82	0.76	99.70
Valdecoxib	40	39.75	0.62	99.36
	50	49.75	0.36	99.50
	60	59.10	0.22	98.51

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

Method Application

The MEKC method validated was applied for the determination of nimesulide and valdecoxib marketed in tablet dosage forms as shown in Table 6, together with the results obtained by the LC method. The experimental values obtained from the two methods were compared statistically by the Student's t-test showing

Table 4. MEKC conditions and range investigated during robustness testing

Variable	Range investigated	Nimesulide[a] (%)	Valdecoxib[a] (%)	Optimized value
Buffer pH	9.65	99.31	100.64	9.75
	9.75	99.40	99.50	
	9.85	100.58	98.90	
Buffer concentration (mM)	34	98.80	98.74	35
	35	99.32	99.43	
	36	98.22	98.67	
SDS concentration (mM)	34	100.74	100.45	35
	35	100.18	100.09	
	36	100.79	101.04	
Percent acetonitrile	4	100.81	100.87	5
	5	99.87	100.16	
	6	99.14	99.53	
Solution stability	Autosampler 48 h 4848 h	101.64	100.81	—
	4–8°C 48 h	100.92	101.76	—

[a]Mean of three replicates.

Table 5. Results of the system suitability test

	Nimesulide[a]				Valdecoxib[a]			
Parameter	Minimum	Maximum	RSD[b] (%)	Status	Minimum	Maximum	RSD[b] (%)	Status
Peak symmetry	1.09	1.14	1.78	Passed	0.95	1.00	1.84	Passed
Theoretical plates	139654	145387	1.81	Passed	86235	90021	1.72	Passed
Migration time	4.21	4.27	0.58	Passed	6.88	7.09	1.15	Passed
Area	15.30	15.97	1.67	Passed	35.59	37.20	1.73	Passed

[a]Values from five replicates.
[b]RS = Relative standard deviation.

Table 6. Comparison between MEKC and LC methods in the analysis of pharmaceutical formulations

			Experimental amount (%)			
	Theoretical amount (mg)		MEKC		LC	
Sample	Nimesulide	Valdecoxib	Nimesulide[a]	Valdecoxib[a]	Nimesulide[a]	Valdecoxib[a]
A	100	40	98.2	100.2	99.2	98.3
B	100	20	100.2	102.5	101.2	100.8
C	100	20	97.5	98.2	98.2	99.4
D	100	10	101.2	104.3	100.5	103.9
E	100	10	103.1	97.2	104.7	98.4

[a]Mean of three replicates.

non-significant difference ($P > 0.05$). The proposed method can be useful for the determination of nimesulide and valdecoxib 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.

CONCLUSION

The results of the validation studies show that the MEKC method is simple, economic, specific, 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 analysis of nimesulide and valdecoxib in pharmaceutical dosage forms, representing, also, an improvement for the quality control, contributing to assure the therapeutic efficacy.

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