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Micellar electrokinetic chromatography for the simultaneous determination of ketorolac tromethamine and its impurities Multivariate optimization and validation

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Abstract

A simple, fast and selective micellar electrokinetic chromatographic (MEKC) method for the simultaneous assay of ketorolac tromethamine and its known related impurities (1-hydroxy analog of ketorolac, 1-keto analog of ketorolac and decarboxylated ketorolac), in both drug substance and coated tablets, is described. The compounds were detected at 323 nm, and flufenamic acid (FL) and tolmetin (TL) were chosen as internal standards to quantify ketorolac tromethamine and impurities, respectively. The multivariate optimization of the experimental conditions was carried out by means of the response surface study, considering as responses the resolution values and analysis time. The optimized background electrolyte (BGE) consisted of a mixture of 13 mM boric acid and phosphoric acid, adjusted to pH 9.1 with 1 M sodium hydroxide, containing 73 mM sodium dodecyl sulfate (SDS). Optimal temperature and voltage were 30 °C and 27 kV. Applying these conditions, all compounds were resolved in about 6 min. The related substances could be quantified up to the 0.1% (w/w) level. Validation was performed, either for drug substances and drug product, evaluating selectivity, robustness, linearity and range, precision, accuracy, detection and quantitation limits and system suitability.
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1. Introduction

Ketorolac ((±)-5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid), a pyrrolizine carboxylic acid derivative structurally related to indomethacin, is a potent and effective nonsteroidal anti-inflammatory drug (NSAID), used principally as analgesic in the short-term management of moderate to severe postoperative pain. It is used intramuscularly, intravenously, orally and as eye drops as the trometamol salt (KT, 1:1 compound with 2-amino-2-(hydroxymethyl)-1,3-propanediol) [1]. Similar to other NSAIDs, the limiting factor for KT is development of gastrointestinal side effects, possibly due to its potent cyclooxygenase inhibitory effect. Because of concerns over the high incidence of reported adverse effects with KT, its dosage and maximum duration of use are restricted. The recommended oral dose in the UK is 10 mg every 4–6 h to a maximum of 40 mg daily for a maximum duration of 7 days [1]. KT is administered by mouth as 2% (w/v) oral drops and 10 mg coated tablets.

According to the information given by the drug substance and drug product producer (Roche, Milan, Italy), three related impurities of ketorolac are known: 1-hydroxy analog (HK), 1-keto analog (KK) and decarboxylated ketorolac (DK). Their structures are shown in Table 1.

Several methods have been described for the analysis of KT. HPLC methods have been developed to determine KT only [2,3], KT and its hydroxylated metabolite [4] and KT enantiomers [5–9] in plasma. Other KT assaying methods in biological fluids include GC-MS [10], electrospray ionization (ESI) MS–MS [11] and voltammetry [12]. As for pharmaceutical matrices, flow injection [13], differential pulse polarography [14] and derivative adsorptive chronopotentiometry [15] have been used for KT assay. Currently, an LC method is the official one reported in the USP 26 for the assay of ketorolac tromethamine drug substance, injection and tablets [16]. As regards the separation and determination of KT related substances, only an HPTLC method has

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Table 1
Chemical structures of ketorolac and related substances

Compound name	Structure
Ketorolac	
(±)-(7-Hydroxy-6,7-dihydro-5H-pyrrolizin-3-yl)-phenyl-methanone (HK)	
5-Benzoyl-2,3-dihydro-pyrrolizin-1-one (KK)	
(6,7-Dihydro-5H-pyrrolizin-3-yl)-phenyl-methanone (DK)	

been described for the quantification of KT, which could be extended to study the drug degradation kinetics in specific acid-base conditions (the expected acid degradation product is HK) [17].

To our knowledge, no method has been described for the assay of KT and the potential impurities (HK, KK, DK). On the other hand, impurity determination is an important issue in pharmaceutical analysis, particularly during quality control. Thus, the aim of this paper was to optimize and validate a micellar electrokinetic chromatographic (MEKC) method able to separate and quantify KT and the above mentioned related substances up to the 0.1% (w/w) level fulfilling the ICH thresholds [18].

Determination of impurities is among the principal roles of capillary electrophoresis (CE) [19–21] within pharmaceutical analysis and represents a challenge to the selectivity of the technique. In fact, the main component and structurally-related impurities often have very similar chemical properties which place great requirements on the necessary selectivity [22–27]. In the present paper, a MEKC method [19–21], based on a micellar system formed by the anionic surfactant sodium dodecyl sulfate (SDS), was developed since the KT related substances are uncharged solutes. The experimental parameters which could influence the separation quality were systematically investigated by means of experimental design [28–36] to achieve the optimum resolutions and analysis time.

Validation of the optimized method was carried out taking into consideration the guidelines for validation of the analytical procedures for drug substances and impurities given by ICH [37].

2. Experimental

2.1. Materials

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

Phosphoric acid was from Sigma–Aldrich (St. Louis, MO, USA). Boric acid and SDS were from Merck (Darmstadt, Germany). Methanol and acetone (HPLC grade) were purchased from Riedel-de Haën (Seelze, Germany).

Working standards of KT and its impurities (HK, KK and DK) and coated tablet excipients were from Roche (Milan, Italy). Lixidol coated tablets (labeled to contain: 10 mg ketorolac tromethamine, lactose, magnesium stearate, titanium dioxide, hypromellose, microcrystalline cellulose and polyethylene glycol 8000) were locally purchased in pharmacies.

Flufenamic acid (FL) and tolmetin sodium salt dihydrate (TL), used as internal standards for the quantification of KT and its impurities, respectively, were from Sigma–Aldrich

(St. Louis, MO, USA), as well as anthracene (AN) used as micelle marker.

Ultrapure water used throughout the study was provided by a Milli-Q system (Millipore/Waters, Milford, MA, USA).

2.2. Solutions

pH 8.0–9.6, 0.01–0.02 M background electrolyte (BGE) buffers examined during the optimization step were prepared by mixing the adequate volume of 0.5 M aqueous solution of boric and phosphoric acids, adjusting pH with 1 M NaOH and filling up to the volume with water. An accurately weighed amount of SDS was then added to the buffer.

Standard stock solutions of KT (5 mg/ml) and of FL (10 mg/ml, internal standard for KT assay) were prepared in methanol. Standard stock solutions of the impurities (0.1 mg/ml each) and of TL (1 mg/ml, internal standard for related substances assay) were prepared in methanol and in water, respectively.

A standard stock solution of anthracene (0.25 mg/ml), micelle marker, was prepared in acetone and was then used as electro-osmotic flow marker. All these solutions were stored at 4 °C and used within 1 week. Working standard solutions were prepared daily by adding the appropriate volume of each of the standard stock solutions directly in a vial and diluting to 500 μl with buffer (13 mM boric acid and phosphoric acid, pH 9.1) in order to achieve the desired final concentrations of the different compounds.

To evaluate the elution time window and the capacity factors of the solutes, 10 μl of anthracene standard stock solution were added to the vial before filling up to 500 μl with buffer.

The solution for tablet assay was prepared as follows: 20 coated tablets were weighed and finely powdered. An accurately weighed portion of the powder, corresponding to about 100 mg of KT, was transferred into a beaker. The content was diluted with 10 ml of methanol, shaken vigorously, sonicated for 15 min, shaken again and then filtered through a dry filter into a 20 ml volumetric flask. The volume was adjusted with methanol, and passed through the beaker and filter. The obtained solution contained KT at a concentration of about 5 mg/ml. 200 μl of this solution were added to a vial, where proper volumes of internal standard solutions were added before filling up to the 500 μl volume with buffer (13 mM boric and phosphoric acids, pH 9.1). The final test concentration of KT was about 2 mg/ml.

2.3. Instrumentation and method conditions

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

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

All CE experiments were carried out on an Agilent Technologies ^{3D}CE system (Agilent Technologies, Waldbronn, Germany) equipped with an on-column diode-array detection (DAD) system. Data acquisition and signal processing were performed using ^{3D}CE ChemStation software (Rev. A.09.01, Agilent Technologies).

Separations were performed on a 48 cm long (39.5 cm effective length) untreated fused silica capillary with an inner diameter of 50 μm and an outer diameter of 375 μm (Composite Metal Services, Hallow, UK) with a detection window built-in by burning off the polyimide coating on the capillary. The UV absorption detector was set at 323 nm, which is near (or actually corresponds to) the maximum wavelength for all the analytes and the internal standards. Samples were injected hydrodynamically by applying a 50 mbar pressure for 5 s. A constant voltage of 27 kV (rise time 0.20 min) was applied with the anode at the inlet and the cathode at the outlet side and the temperature was held constant at 30 °C. The standard run buffer consisted of an aqueous solution of 13 mM boric and phosphoric acids, adjusted to pH 9.1 with 1 M NaOH, containing 73 mM SDS.

Before use, a new capillary was flushed with 1 M NaOH and water for 5 min each. Between two runs, the capillary was flushed with water (1 min), 0.1 M NaOH solution (1 min), water (2 min) and run buffer (2 min). To improve repeatability of migration times, buffer vials were replenished after each injection.

2.4. Calibration curves

Calibration curves were obtained by plotting the peak area/migration time ratio of each analyte divided by the peak area/migration time ratio of the respective standard versus each analyte/internal standard concentration ratio. FL was used as internal standard for KT assay, while TL was used as internal standard for the assay of KT impurities.

The curves for drug substances and drug product were evaluated for KT at around 40–120% of test concentration (2 mg/ml). For HK, KK and DK the same curves were evaluated over the range 0.1–1% corresponding to a working concentration of 2–20 μg/ml. The values of internal standard concentrations were fixed, respectively, to 1 mg/ml for FL and to 20 μg/ml for TL and were held constant throughout all the experiments.

For drug substance, five different concentrations of each analyte, together with its internal standard, were prepared by adding the appropriate volumes of the standard stock solutions to different vials and diluting to 500 μl with buffer. For drug product, five separate weighings of synthetic mixtures of the components were used. Each solution was analyzed twice.

2.5. Experimental design

The NEMROD-W software package [38] was used to generate experimental designs and to perform statistical analysis of the data.

A 25-run *D*-optimal design was used to find optimal conditions and the experiments were carried out in a random-

ized order with KT concentration of 2.4 mg/ml, HK, KK and DK concentrations of 20 μg/ml, FL concentration of 1 mg/ml and TL concentration of 20 μg/ml.

An 8-run Plackett–Burman design was used to test robustness. The experiments were run in a randomized order with KT concentration of 2 mg/ml, HK, KK and DK concentrations of 11 μg/ml, FL concentration of 1 mg/ml and TL concentration of 20 μg/ml.

3. Results and discussion

3.1. Method development

Ketorolac is a weak acid ($pK_a = 3.49$) and in the studied pH range (8.0–9.6), it is in the anionic form, while its three impurities (HK, KK and DK) are neutral compounds. Thus, the simultaneous assay of these analytes required the use of MEKC. In this operational mode, micelles added to a buffer solution form, with the neutral compound, a charged "complex" with an effective electrophoretic mobility [21]. The differential partition of the uncharged species in the retentive phase obtained gives rise to the separation due to the differential migration rates.

Nature and concentration of buffer and surfactant, buffer pH, instrumental parameters such as temperature and applied voltage can all significantly influence MEKC analyses and should be incorporated in the method development strategy. Moreover, these important parameters are often interactive in nature. As a result, to make the separation of the mixture of target compounds successful, all these factors should be optimized and the most efficient way to achieve this target is to use a multivariate strategy such as experimental design, where the effects of the considered factors on the selected responses are evaluated simultaneously [28–30].

The performances required by the MEKC method for the determination of KT and its related substances included baseline separation of the compounds, impurities quantitation levels of 0.1% (w/w) or less, and short analysis time. The choice of internal standard for the assaying took into consideration the high difference in the expected concentrations of KT versus its impurities and consequently in the measured absorbances. Thus, two internal standards with absorption characteristics similar to those of analytes, but with different concentration levels, were selected. In particular, FL at a concentration level of 1 mg/ml was selected as KT internal standard and TL at a concentration level of 20 μg/ml was selected as the impurities internal standard.

Preliminary experiments showed that SDS was a good choice as surfactant and among alkaline buffers (pH 7.5–10.0) a suitable BGE was chosen. Britton–Robinson buffer, borate buffer, phosphate buffer, borate/phosphate buffer and AMPSO buffer were tested and the most promising results were obtained with borate/phosphate buffer. This latter offered good selectivity as regards the considered problem, and in general had a good buffering capacity in a quite large pH interval, making it possible to increase the buffer concentration and to add the desired SDS amount without an excessive rise in the measured current.

A multivariate optimization was carried out with the aim of obtaining a rapid and complete separation of all compounds. In order to carry out an in-depth study of the problem, the response surface methodology (RSM) was used. In fact, the purpose of RSM is to obtain a mathematical model, generally empirical, for each response, which adequately represents changes in the response within the zone of interest. In this way, it is possible to predict from the input variables, the response over the whole domain and to know how the effects of one factor will be influenced by changes in the levels of another [29].

In this case, the selected factors were voltage (V, U_1), borate/phosphate concentration (buffer conc., U_2), buffer pH (pH, U_3), SDS concentration (SDS conc., U_4) and temperature (T, U_5). Preliminary experiments showed that the migration order of the analytes was: KT, TL, FL, HK, KK and DK. As expected the anionic compounds (KT, TL and FL) had a migration time shorter than the neutral compounds due to the different affinity for the negative pseudo-stationary phase. Among the neutral compounds, the polarity and lipophilicity determined the interaction with SDS micelles. HK, due to the presence of an hydroxy group, had a lower migration time than KK and DK, and DK was the last migrating analyte. Thus, in the multivariate optimization the considered responses were the DK migration time, corresponding to the analysis time (to be minimized) and the resolution (to be maximized) of the following peak pairs, calculated accordingly to USP 26 [16]: KT and TL (R_1); HK and KK (R_4); KK and DK (R_5). The resolutions among FL and the other analytes (TL and HK) were not considered, as they were not critical.

The current generated was not included as a modeled response, although this factor was kept under control throughout the experiments.

The choice of experimental domain for each factor (Table 2) was made on the basis of preliminary studies, taking into account mainly analysis speed and current level. As regards the domain of SDS concentration, it was observed that using SDS concentrations below 30–35 mM, the impurities peak shapes were strongly influenced by the concentration of KT. This means that, when the concentration of KT was as low as the impurities concentration (about 10 μg/ml), the peak shapes of HK, KK and DK were

Table 2
Experimental domain of the factors during response surface study

Factor	Experimental domain
U_1, V (kV)	24–30
U_2, buffer conc. (mM)	10–20
U_3, pH	8.0–9.6
U_4, SDS conc. (mM)	50–80
U_5, T (°C)	24–30

satisfactory. Instead, when KT was analyzed at the test concentration (2 mg/ml), the peak shapes of the impurities worsened and/or peak splitting occurred. Thus, we decided to study the factor SDS concentration above 50 mM, where this effect was avoided.

The response surface for each considered response was approximated by a second-order polynomial function:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^4 + \beta_{55} x_5^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{15} x_1 x_5 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{25} x_2 x_5 + \beta_{34} x_3 x_4 + \beta_{35} x_3 x_5 + \beta_{45} x_4 x_5 + \varepsilon$$

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.

A *D*-optimal design [28,29] was employed to select from a Doehlert design [29] a minimum number of experiments which enabled an accurate estimate of the model coefficients. A 25-run matrix, with three replicates at the center of the experimental domain, was the best compromise between number of experiments and quality of information [28].

All 25 experiments were run in a day, at fixed concentration values above the test concentration level, in order to avoid possible resolution problems during the routine analysis. In this way it was possible to always assure a sufficient resolution among all the compounds in the whole calibration range.

The experimental matrix and the measured responses are reported in Table 3.

The regression model for each considered response was tested through analysis of variance (ANOVA) to determine its significance and validity [28,29]. All models were found significant and valid, thus indicating that the observed response changes were due to a level change of factors and that the model gave a good description of the experimental data [28,29].

The response surfaces were drawn, evaluating the effects of two factors at a time and setting the other three at their central values. Observing the surfaces obtained plotting pH versus SDS conc., shown in Fig. 1, one can easily deduce that to speed up the analysis (t) pH had to be set at a high value (Fig. 1a) while the effect of SDS conc. is not important. In fact, the migration time strongly decreases moving from low pH to high pH both for high values and low values of SDS conc. As concern the dependence of resolutions from these two factors, it is easy to see from Fig. 1b–d, that when the separation between two anionic compounds (R_1, KT/TL) is involved the SDS conc. is more important than the pH value. From Fig. 1b it appears that the resolution value (R_1, KT/TL), when SDS conc. is at its high level, is high at all pH values. On the other hand, when the considered response is the resolution between two neutral compounds

Table 3
Twenty-five-run *D*-optimal experimental matrix

U_1	U_2	U_3	U_4	U_5	t (min)	R_1	R_4	R_5
1.0000	0.0000	0.0000	0.0000	0.0000	6.015	1.57	3.27	3.17
0.5000	0.8660	0.0000	0.0000	0.0000	6.822	1.97	3.37	3.71
0.5000	−0.8660	0.0000	0.0000	0.0000	5.786	0.95	2.42	2.20
−0.5000	0.8660	0.0000	0.0000	0.0000	8.684	2.12	3.03	2.90
0.5000	0.2887	0.8165	0.0000	0.0000	5.422	2.13	3.10	3.02
−0.5000	−0.2887	−0.8165	0.0000	0.0000	12.739	1.41	3.49	3.17
−0.5000	0.2887	0.8165	0.0000	0.0000	6.968	2.22	2.56	2.44
0.0000	−0.5774	0.8165	0.0000	0.0000	5.508	1.39	2.10	1.85
0.5000	0.2887	0.2041	0.7906	0.0000	6.352	2.95	2.96	3.01
−0.5000	−0.2887	−0.2041	−0.7906	0.0000	7.478	0.62	3.04	2.98
0.0000	0.5774	−0.2041	−0.7906	0.0000	7.572	0.96	3.87	4.01
−0.5000	0.2887	0.2041	0.7906	0.0000	8.132	3.16	2.56	2.35
0.0000	−0.5774	0.2041	0.7906	0.0000	6.567	2.33	2.25	2.04
0.0000	0.0000	−0.6124	0.7906	0.0000	10.542	2.46	2.92	2.87
0.5000	0.2887	0.2041	0.1581	0.7746	5.779	1.80	3.04	3.07
0.5000	−0.2887	−0.2041	−0.1581	−0.7746	6.902	1.27	3.03	2.90
0.0000	0.5774	−0.2041	−0.1581	−0.7746	8.704	1.97	3.54	3.33
0.0000	0.0000	0.6124	−0.1581	−0.7746	6.455	1.77	2.77	2.40
0.0000	0.0000	0.0000	0.6325	−0.7746	7.920	2.69	2.67	2.32
−0.5000	0.2887	0.2041	0.1581	0.7746	7.291	1.94	2.62	2.57
0.0000	0.0000	−0.6124	0.1581	0.7746	9.241	1.51	3.59	3.40
0.0000	0.0000	0.0000	0.0000	0.0000	7.422	1.57	2.93	2.79
0.0000	0.0000	0.0000	0.0000	0.0000	6.998	1.49	2.55	2.55
0.0000	0.0000	0.0000	0.0000	0.0000	6.936	1.53	2.85	2.70
0.0000	0.0000	0.0000	0.0000	0.0000	7.016	1.58	3.01	2.85

Factors: U_1, voltage; U_2, borate/phosphate concentration; U_3, pH; U_4, SDS concentration; U_5, temperature. Responses: analysis time (t, measured as DK migration time in minutes), KT/TL resolution (R_1), HK/KK resolution (R_4), KK/DK resolution (R_5).

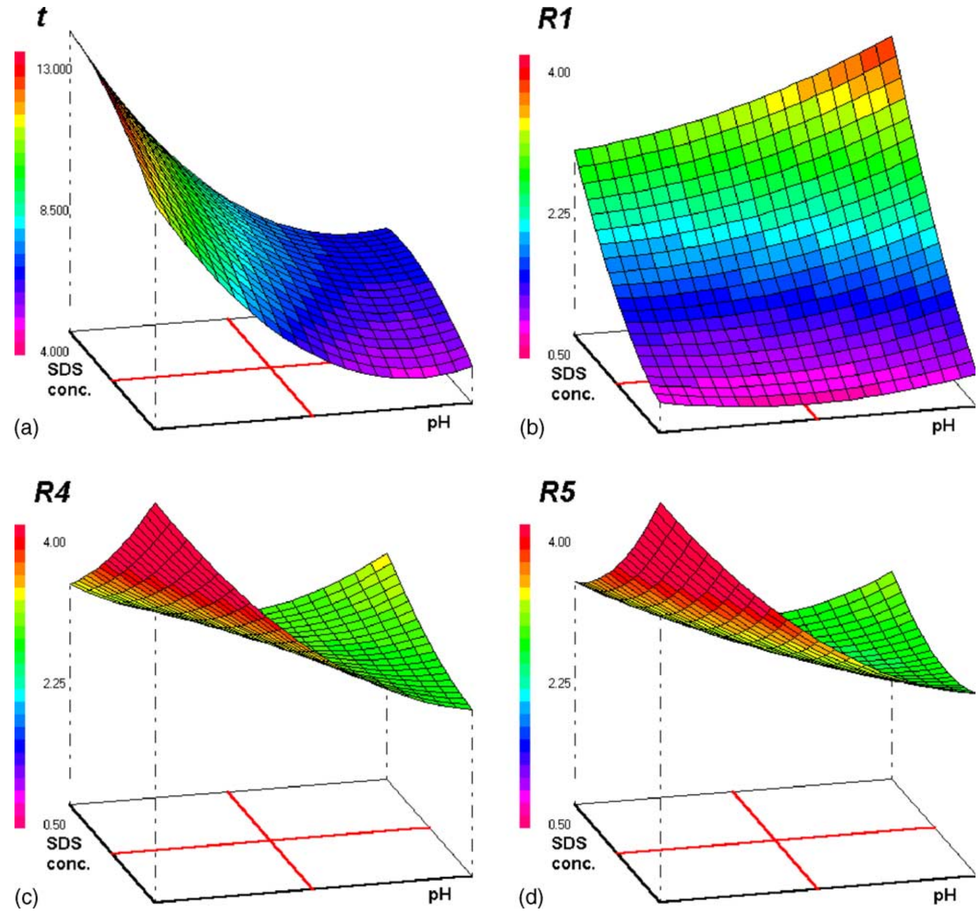

Fig. 1. Response surfaces obtained plotting pH vs. SDS concentration (SDS conc.): (a) analysis time (t) response surface; (b) KT/TL resolution (R_1) response surface; (c) HK/KK resolution (R_4) response surface; (d) KK/DK resolution (R_5) response surface.

(R_4, HK/KK; R_5, KK/DK), it is important the interaction between the two factors (pH and SDS conc.). In particular, a positive interaction exists between these two factors and in order to maximize R_4 (Fig. 1c) and R_5 (Fig. 1d) pH and SDS conc. should be set at the low level of the considered experimental domain. Concerning the dependencies of the considered responses from the other factors, that is voltage, temperature and buffer concentration (results not shown) it is possible to state, as expected, that analysis time decreases by increasing the voltage and temperature and by decreasing the buffer concentration. For the responses R_1, R_4 and R_5, buffer concentration is significant and in order to maximize these responses it should be set at its high level. Temperature and voltage influence the responses R_4 and R_5 and should be set at their high value.

Summarizing, due to the number of responses considered and due to the fact that the conditions required to optimize a response may often be in conflict with the values needed to optimize another response, the optimum conditions cannot be easily found by directly observing the response surfaces. A way of overcoming this difficulty is the Derringer's desirability function [29,32,33,39]. In this approach each response is associated with its own partial desirability function which may vary from 0 to 1, according to the closeness of the response to its target value. The individual desirability functions are then combined together, as the geometric mean, to obtain the overall desirability function (D) whose maximum value can then be looked for within the domain.

NEMROD-W software was used to define the partial desirability functions, to find the overall function D and to select the optimal point fulfilling all optimization criteria ($D = 1$). The selected optimal conditions corresponded to: U_1, voltage, 27 kV; U_2, phosphate/borate concentration, 13 mM; U_3, pH, 9.1; U_4, SDS concentration, 73 mM; U_5, temperature, 30 °C. The graphical representation of D is depicted in Fig. 2, where D is shown for two factors at a time, setting the other three at their optimized values.

Each model found was then validated to check its ability in the prediction. The confidence interval around the optimized conditions [29], for each modeled response at a probability level of 99%, was calculated using the standard deviation obtained from the replicates (t, S.D. = 0.222 min; R_1, S.D. = 0.04; R_4, S.D. = 0.20; R_5, S.D. = 0.13); the predicted response using the optimized conditions was used as mean value. The confidence intervals were 5.948 ± 0.648, 2.04 ± 0.12, 2.59 ± 0.59, 2.44 ± 0.38 for t, R_1, R_4 and R_5, respectively. The observed experimental responses (t, 5.838; R_1, 1.94; R_4, 2.64; R_5, 2.35) were inside the relative confi-

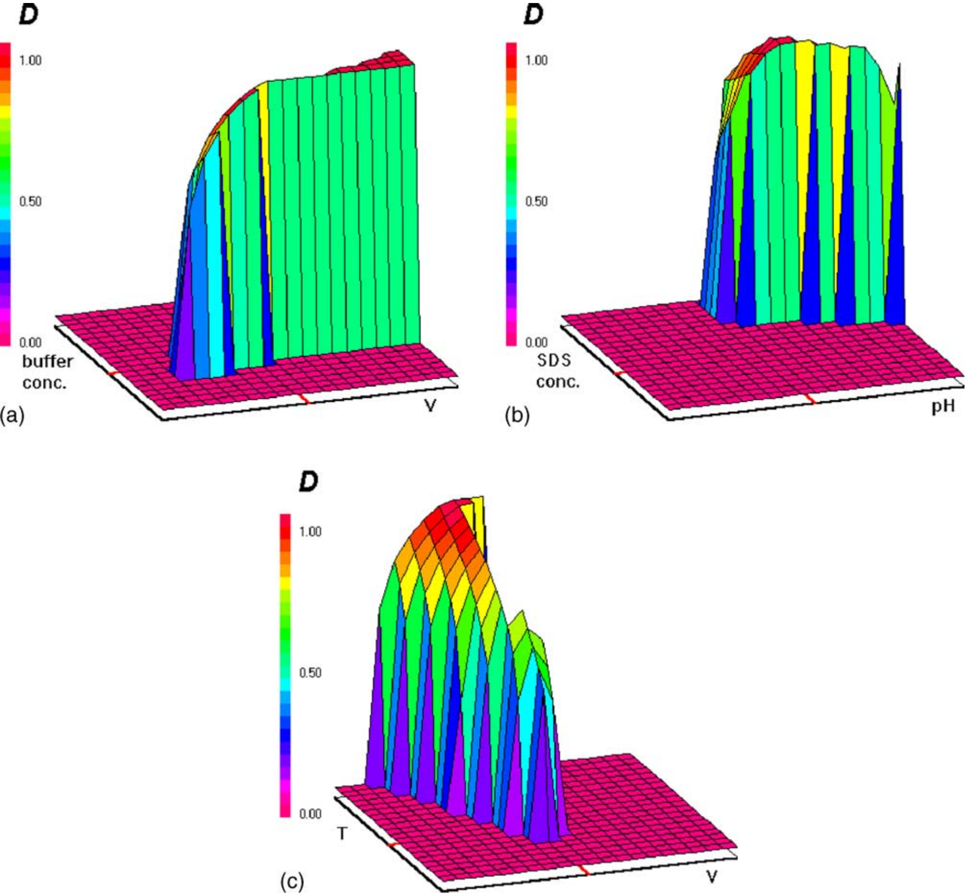

Fig. 2. Desirability function three-dimensional plots obtained by plotting: (a) voltage (V) vs. borate/phosphate concentration (buffer conc.); (b) pH vs. SDS concentration (SDS conc.); (c) voltage (V) vs. temperature (T).

dence intervals, thus showing that there was a good agreement between predicted and measured responses. Applying the optimized conditions, all compounds were resolved in about 6 min with a current generated of about 70–75 μA.

In Fig. 3, a typical electropherogram obtained under optimal conditions is reported when KT is at the nominal concentration (2 mg/ml) and the impurities are in the middle of the linearity range (0.55% (w/w), corresponding to a working concentration of 11 μg/ml).

3.2. MEKC parameters

From the retention behavior of the solutes, applying the optimized conditions, further conclusions with respect to the

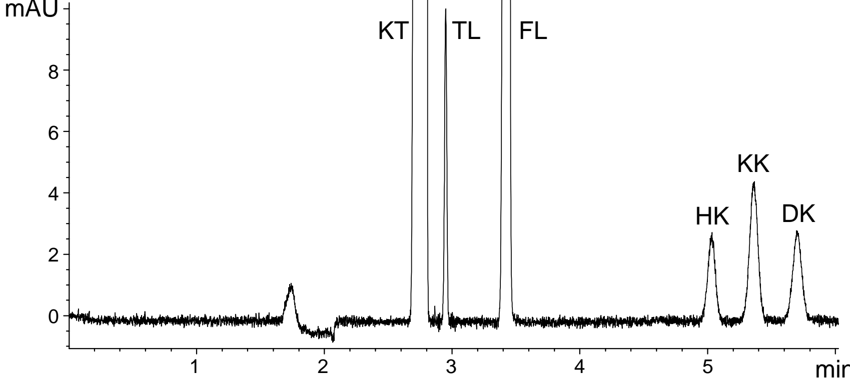

Fig. 3. Electropherogram of KT and related substances referring to optimal conditions: V, 27 kV; BGE conc., 13 mM; pH, 9.1; SDS conc., 73 mM; T, 30 °C. Hydrodynamic injection: 50 mbar, 5 s. Detection wavelength: 323 nm.

physical and chemical properties of the analytes, such as the extent of solute association with micelles, can be drawn.

In MEKC, the separation of neutral and weakly ionized solutes is essentially chromatographic, and migration of the solute can be rationalized in terms of the capacity factor of the solute (k'), which is given by [40,41]:

$$k' = \frac{(t_m - t_0)}{t_0\,(1 - t_m/t_{mc})}$$

where t_m is the migration time of the solute, t_0 the migration time of the unretained solute moving at the electroosmotic flow (EOF) rate, and t_{mc} is the micelle migration time [40,41]. On the other hand, for charged analytes, the migration phenomenon does not only involve the partitioning mechanism between the aqueous phase and the micellar phase, but also their electrophoretic migration. In such a case, the calculation of the partition coefficient requires the determination of the corrected capacity factor (k'_{corr}) defined according to the following relationship [41]:

$$k'_{corr} = \frac{(t_m - t'_m)}{t'_m(1 - t_m/t_{mc})}$$

where t_m and t'_m are the migration times of the solute in MEKC and capillary zone electrophoresis (CZE) conditions, respectively, and t_{mc} the micelle migration time. This formula is correct only for identical electro-osmotic mobility values in MEKC and CZE modes.

In this case, the electro-osmotic mobility changed by about 5.1% between MEKC and CZE conditions; t_0 was determined by injecting acetone as an electroosmotic marker which does not enter the micelle, while t_{mc} was measured by injecting the micelle marker anthracene which migrates while continuously absorbed in the micelle. The analyses were performed at 250 nm, and for KT and TL an inversion in the migration order was observed passing from MEKC mode to CZE mode. The confidence intervals ($n = 5, \alpha/2 = 0.05$) of k' and k'_{corr} are the following: KT, 0.38 ± 0.03; TL, 0.56 ± 0.03; FL, 1.10 ± 0.04; HK, 13.70 ± 0.27; KK, 25.30 ± 0.68; DK, 89.88 ± 8.32. These results are in agreement with the molecule polarity and lipophilicity. In fact, KT and internal standards, at pH 9.1 are in the anionic form, thus their retention in the negative micelles is weak. While for the neutral compounds, the decrease of polarity determines an increase of the affinity for the pseudostationary phase. This is demonstrated by the value of k'_{corr} that for the impurities increases moving from HK to DK.

3.3. Method validation

When a CE method is employed for quality control of pharmaceuticals, its performance characteristics fall into the same category as those of LC and GC techniques. Thus, the validation principles for chromatographic method also apply to CE methods [21]. The developed method was validated according to the ICH guidelines [37] using KT and impurities working solutions. The procedure required the assessment of selectivity, robustness, linearity and range, accuracy, precision, detection and quantitation limits and system suitability. The test concentrations for validation were 2 mg/ml for KT (nominal concentration) and 11 μg/ml for the impurities (middle of the linearity range, 0.55% (w/w)).

FL (1 mg/ml) was used as internal standard for KT determination, while TL (20 μg/ml) was chosen for the determination of impurities.

3.3.1. Selectivity

Selectivity of the method was assessed by analyzing test solutions separately spiked with each of the compounds and additionally by analyzing standards of each analyte individually. The peak identity was also confirmed by the spectra using DAD. All the peaks proved to be baseline separated, and, applying the optimized conditions, the confidence interval ($n = 4, \alpha/2 = 0.005$) of the resolution values for the critical adjacent peaks were the following: R_1 (KT/TL), 2.37 ± 0.55; R_4 (HK/KK), 2.86 ± 0.59; R_5 (KK/DK), 2.63 ± 0.49.

To demonstrate freedom from interference in the drug product, the tablet excipients were analyzed using the described method and an electropherogram absolutely free of any peaks was obtained.

3.3.2. Robustness

During robustness testing, a method must prove to be able to remain unaffected by small, but deliberate variations in method parameters, thus showing its own reliability during normal usage [16,32,37,42,43]. It is advisable to simultaneously study the possible variations of method parameters in an interval chosen symmetrically around the optimized conditions. This interval represents the variations expected during method transfer.

In this case, the five selected parameters were the same considered in the optimization step and Table 4 reports their experimental domain. An 8-run Plackett–Burman matrix, able to identify the main effects of the factors, was used [29].

The effects of the five factors were assessed on the following responses which were considered appropriate for describing the quality of the separation: R_1 (KT/TL), R_4 (HK/KK) and R_5 (KK/DK). Graphic analysis of effects [44] allowed the significant effects to be detected. None of the factors studied had a significant effect on R_1 and R_4. Instead, as regards R_5, voltage (U_1) and BGE concentration (U_2) were identified as critical parameters. Both of them

Table 4
Experimental domain of the factors during robustness testing

Factor	Experimental domain
U_1, V (kV)	26–28
U_2, buffer conc. (mM)	12–14
U_3, pH	9.0–9.2
U_4, SDS conc. (mM)	72–74
U_5, T (°C)	29–31

Table 5
Linearity data obtained for KT and KT related substances ($n = 5$, $k = 2$)

Analyte	Concentration range (mg/ml)	Slope	Slope (S.D.)	Intercept	Intercept (S.D.)	Standard error	R^2	R^2_{CV}
Drug substance								
KT	0.8–2.4	1.987	0.022	0.1318	0.037	0.012	0.9991	0.9985
HK	0.002–0.020	0.9817	0.014	−0.010	0.009	0.005	0.9983	0.9969
KK	0.002–0.020	1.7101	0.027	−0.053	0.017	0.009	0.9980	0.9968
DK	0.002–0.020	1.0675	0.017	−0.0429	0.011	0.005	0.9981	0.9972
Drug product								
KT	0.8–2.4	1.9978	0.020	−0.0886	0.033	0.011	0.9992	0.9988
HK	0.002–0.020	0.8522	0.014	0.0100	0.009	0.005	0.9978	0.9968
KK	0.002–0.020	1.5645	0.025	−0.0260	0.016	0.008	0.9980	0.9970
DK	0.002–0.020	0.9798	0.020	−0.0335	0.013	0.006	0.9967	0.9948

had a positive effect on R_5, even if the separation quality was always good. As a consequence, it was necessary to set a precautionary statement about carefully controlling voltage and BGE concentration.

3.3.3. Linearity

Linearity was assessed for both drug substances and drug product. For drug product, a calibration curve was prepared using a placebo tablet solution.

Calibration graphs for KT were linear within the studied range of 40–120% of the nominal test concentration. The same holds true for the calibration graphs of the related substances within the studied range of 0.1–1.0% (w/w) of the nominal KT concentration.

Linearity data obtained are reported in Table 5.

3.3.4. Migration time and peak area repeatability

Within-day precision and between-day precision of migration times and corrected peak areas ratios was evaluated performing six replicated injections of KT and related substances each day over a 3-day period. The results (Table 6) show that R.S.D. values obtained for between-day precision were in the same order of magnitude as those obtained for within-day precision.

The use of internal standard was strictly necessary in order to compensate for the poor precision observed with the hydrodynamic injection. A good method precision regards to corrected area ratios was achieved.

On the other hand, the use of vial replenishment with fresh BGE after each injection was fundamental to improving precision data regarding migration time.

3.3.5. Accuracy and precision

The same procedure has been followed to evaluate these parameters for KT and for KT impurities. These parameters were assessed for each analyte at three concentration levels (three replicates each) covering the linearity range.

In the case of the assay of the analytes as drug substances, accuracy was determined applying the analytical method to standards of known purities. As for the assay in a formulated product, accuracy was determined by application of the analytical method to synthetic mixtures of the drug product components to which known amounts of analytes have been added within the linearity range of the method.

The evaluation of accuracy and precision for KT and its related impurities was performed simultaneously and the obtained results are reported in Table 7.

3.3.6. Limit of detection (LOD) and limit of quantitation (LOQ)

The baseline noise was calculated by Agilent Technologies ChemStation software and signal to noise (S/N) values were determined dividing each peak height by the noise. For each of the three impurities, LOD and LOQ were then determined from three and ten times this value, respectively, and are listed in Table 8.

Table 6
Precision data for the migration time and peak area ratio for a sample of 2 mg/ml KT and 11 μg/ml KT related substances

	R.S.D. ($n = 6$) (%)			R.S.D. ($n = 18$) (%) between Days 1–3
	Day 1	Day 2	Day 3	
Corrected peak areas ratios (analyte to internal standard)				
KT (%)	0.21	0.27	0.14	0.20
HK (%)	2.97	2.92	1.48	2.74
KK (%)	2.87	3.75	4.17	3.84
DK (%)	2.83	1.31	2.89	6.77
Analysis time (%)	1.62	0.89	1.95	1.97

Table 7
Accuracy and precision data for the assay of KT and KT related substances in drug substances and drug product ($n = 3$, $\alpha/2 = 0.025$)

Analyte	Concentration level (mg/ml)	Drug substance		Drug product	
		Accuracy (recovery, %)	Repeatability (R.S.D., %)	Accuracy (recovery, %)	Repeatability (R.S.D., %)
KT	0.9	101.6 ± 0.9	0.4	101.8 ± 1.8	0.7
	2	98.7 ± 0.4	0.2	101.6 ± 0.6	0.3
	2.3	99.5 ± 1.2	0.5	101.0 ± 2.0	0.8
HK	0.003	102.8 ± 12.0	4.7	103.5 ± 8.9	3.5
	0.011	97.2 ± 5.7	2.4	101.9 ± 3.2	1.3
	0.019	99.2 ± 9.7	3.9	105.1 ± 10.0	3.8
KK	0.003	106.5 ± 7.5	2.8	104.3 ± 8.6	3.3
	0.011	95.9 ± 8.9	3.7	99.8 ± 10.1	4.1
	0.019	100.7 ± 7.0	2.8	103.2 ± 4.9	1.9
DK	0.003	104.7 ± 10.7	4.1	101.9 ± 9.1	3.6
	0.011	96.7 ± 8.0	3.3	104.1 ± 7.9	3.0
	0.019	101.9 ± 4.8	1.9	103.6 ± 13.9	5.4

Table 8
LOD and LOQ values and validation of LOQ values

Analyte	LOD (μg/ml)	LOQ (μg/ml)	R.S.D. ($n = 8$, LOQ), drug substance (%)	R.S.D. ($n = 8$, LOQ), drug product (%)
HK	1	2	6.0	4.1
KK	0.6	1.2	6.6	6.0
DK	0.9	1.8	4.6	5.2

LOQ values were then validated both for drug substances and drug product, performing eight injections at the LOQ concentration values and evaluating the obtained corrected area ratio R.S.D. (Table 8).

3.3.7. System suitability

To ensure that the validity of the analytical method is maintained whenever used, a series of system suitability parameters was established as a consequence of robustness test, choosing the lowest and highest values obtained for each response: $1.94 < R_1 < 2.48$; $2.57 < R_4 < 3.21$; $2.35 < R_5 < 3.03$. These values define the minimum performance criteria that this system must meet prior to usage.

Moreover, it has been suggested as being good working practice to include an indication of the expected level of current as system suitability type check [23]. In this case, the current expected range is 64–82 μA. This range was deduced from the values of current observed in the experiments run for robustness test as well.

R.S.D. for replicate injections did not exceed the values given in EP4 [40].

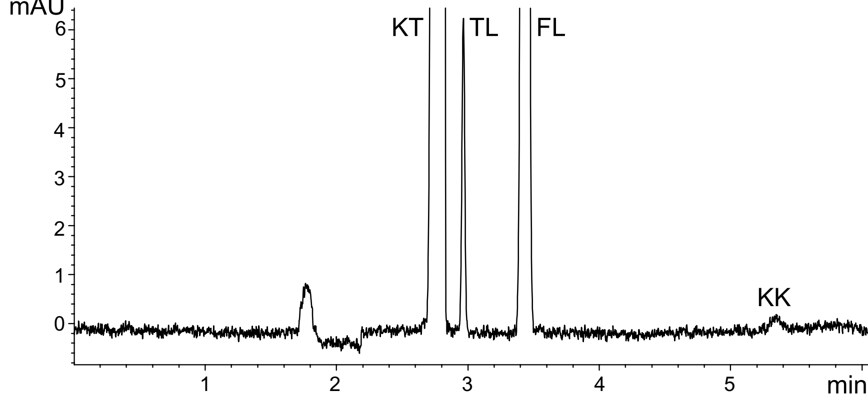

Fig. 4. Electropherogram of a real sample of Lixidol tablets referring to optimal conditions: V, 27 kV; BGE conc., 13 mM; pH, 9.1; SDS conc., 73 mM; T, 30 °C. Hydrodynamic injection: 50 mbar, 5 s. Detection wavelength: 323 nm.

3.4. Applications

The developed method was applied to the assay of Lixidol tablets and the electropherogram of the real sample analyzed is reported in Fig. 4.

The recovery of KT was in agreement with the 10 mg declared content ($n = 3, \alpha/2 = 0.025$, recovery 98.2±0.6%, R.S.D. 0.2%). As regards the determination of KT related substances, HK and DK were not detected, while KK was detected at concentration levels near the LOD (0.6 μg/ml).

4. Conclusions

A simple and fast MEKC method was developed and found efficient for the assay of ketorolac tromethamine and its potential related substances in bulk drug and coated tablets. In the optimization step, an experimental design strategy was applied making it possible to achieve good results in terms of quality of separation and analysis time. All peaks were baseline separated and the analysis was performed in about 6 min. Satisfactory method performances have been demonstrated through the validation process. Potential impurities can be reliably verified and quantified at 0.1% (w/w) of the bulk drug, thus the presented MEKC method may be successfully applied in pharmaceutical quality control.

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