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Research Article

Mixture design in the optimization of a microemulsion system for the electrokinetic chromatographic determination of ketorolac and its impurities: Method development and validation

Microemulsion EKC (MEEKC) was used for the determination of ketorolac and its three impurities. The microemulsion system was optimized, for the first time in the literature, using a multivariate strategy involving a mixture design. A 13-run experimental plan covering an experimental domain defined by the components aqueous phase (10 mM borate buffer pH 9.2), oil phase (*n*-heptane) and surfactant/cosurfactant (SDS/*n*-butanol) was carried out. Good results were obtained with all microemulsions tested considering as responses analysis time and resolution, and according to the desirability function the best microemulsion system was constituted by 90.0% 10 mM borate buffer, 2.0% - *n*-heptane, 8.0% of SDS/*n*-butanol in 1:2 ratio. Finally, with the aim of reducing analysis time, a response surface study was carried out in the experimental domain defined by the process variables temperature and voltage and the best values were 17°C and −17 kV, respectively. Applying the optimised conditions, a complete resolution among the analytes was obtained in about 3 min using the short-end injection method. The method was validated for both drug substances and drug product and was applied to the quality control of ketorolac in coated tablets. A comparison of MEEKC, MEKC and CEC for assaying ketorolac and its related substances has been made.

Keywords: Impurities / Ketorolac / Microemulsion electrokinetic chromatography
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1 Introduction

Microemulsion EKC (MEEKC) is an operative mode of CE where microemulsion droplets are regarded as the pseudostationary phase and can be used to influence the separation behavior of analytes [1–3]. MEEKC offers the possibility of highly efficient separations of both charged and neutral solutes covering a wide range of water solubilities [4, 5]. In addition, MEEKC can be applied to a wider range of solutes than MEKC since solutes are more easily able to penetrate the surface of the droplet than the surface of a micelle which is much more rigid [4, 5].

Microemulsions used in MEEKC are usually oil-in-water. Nanometer-sized oil droplets are suspended in an aqueous buffer and are prepared by mixing oil, water, surfactant/cosurfactant (S/CoS) [6]. In general, microemulsions require specific ratios of the respective components, which can only be varied within a very narrow range in order to maintain the system's stability [7]. A stable homogeneous mixture is transparent and the transparent-to-turbid state can be brought about by variations in temperature, pressure or composition of the mixture [8].

Several studies investigating the suitable oil phase, S/CoS were carried out in order to achieve optimum separations [5, 9–12]. Once the type of buffer, oil phase, S/CoS have been selected, specific combinations of

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Abbreviations: ANOVA, analysis of variance; **CCD**, central composite design; **DAD**, diode-array detector; **DK**, ketorolac decarboxylated; **HK**, 1-hydroxy analog of ketorolac; **KK**, 1-keto analogue of ketorolac; **KT**, ketorolac; **MEEKC**, microemulsion EKC; **S/CoS,** surfactant/cosurfactant; **TL**, tolmetin

them are required to form microemulsions. Different ratios of the components can be tested to obtain the optimum system for a specified analysis. In fact, the complexity of the microemulsion composition and of the MEEKC separation process allows great manipulations to be made during method development in order to achieve the desired selectivity.

Several papers describing the use of experimental design for method optimization have been published in the field of CZE [13–18] and MEKC [19–23] method development. While, to our knowledge, only two papers involving a multivariate optimization of MEEKC methods have been published [24, 25], no paper regarding the use of a multivariate strategy involving mixture design to find the optimum microemulsion buffer for EKC has been reported yet.

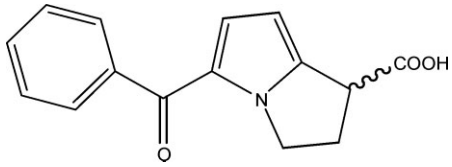
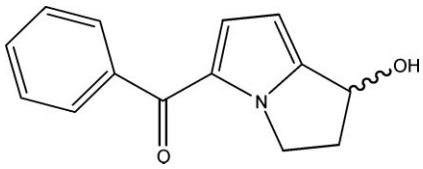
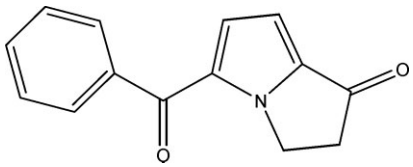
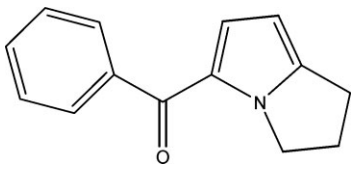
According to literature, today experimentation in this field is still done by changing the value of one factor (variable) at a time in an unsystematic way in order to find the optimum conditions of a complex system. This is not an efficient and rational strategy; in fact changing one factor at a time does not give any information about the position of the real optimum in the common case where there are interactions between factors. Other common problems associated with the univariate approach are that it unnecessarily performs many runs, and does not provide mapping of the experimental space [26].

On the other hand, the most important aspects of statistical experimental design include fewer trials, detection of interactions between factors, detection of optima and model-building from the results [26, 27].

In particular, mixture design [28–30] is suitable for blending problems [30–35] and in this paper it was used to find an optimum microemulsion system for MEEKC analysis of ketorolac (KT) and its three neutral potential impurities.

KT, a pyrrolizine carboxylic acid derivative structurally related to indomethacin, is a potent and effective nonsteroidal anti-inflammatory drug, used principally as analgesic in the short-term management of moderate to severe postoperative pain [36]. There are three known impurities of KT according to the information given by the drug producer, Roche, and the current USP [37]: 1-hydroxy analogue (HK), 1-keto analogue (KK) and decarboxylated ketorolac (DK) (Table 1). For the determination of KT and its impurities only MEKC [19] and CEC [38] methods have recently been developed. Both the meth-

Table 1. Structural formulas of ketorolac and related substances

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

ods were suitable for quality control of the drug. CEC made it possible to finely modulate the method selectivity, while MEKC was easier and cheaper to perform, as it did not require packed capillaries and long preconditioning. However, few studies have been published about impurity determination with MEEKC [39, 40]. Thus, with the aim of providing additional data on the potential of MEEKC for the quality control of drugs, a new MEEKC method for KT and related substances determination was developed. Method validation was performed according to ICH guidelines [41], for both drug substance and drug product.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals used in this study were of analytical reagent grade with no further purification. Sodium borate was from BDH Laboratory Supplies (Poole, UK), methanol and acetone (both HPLC grade), *n*-heptane and sodium dodecyl sulfate (SDS) were from Sigma-Aldrich (St. Louis, MO, USA). *n*-Butanol was purchased from Merck (Darmstadt, Germany). Working standards of KT and its impurities (HK, KK, DK) and coated tablet excipients (lactose, magnesium stearate, titanium dioxide, hydroxypropylmethylcellulose, microcrystalline cellulose and PEG 8000) were kindly donated by Roche (Milan, Italy). Lixidol® coated tablets, labelled to contain 10 mg of KT, were locally purchased in pharmacies. Tolmetin sodium salt dihydrate (TL), internal standard and anthracene (AN), microemulsion marker, were obtained from Sigma-Aldrich. Ultrapure water was used throughout the study.

2.2 Solutions, microemulsion and sample preparation

Standard stock solutions of KT (5 mg/mL) and TL (internal standard, 10 mg/mL) were prepared in water, and standard stock solutions of HK, KK and DK (0.1 mg/mL each) were prepared in methanol. A standard stock solution of anthracene (0.25 mg/mL), microemulsion marker, was prepared in acetone, which constituted the EOF marker. All these solutions were stored at 4°C and used within 1 wk. Working standard solutions were prepared daily by diluting standard stock solutions with 10 mM sodium borate directly in a vial to 500 μL in order to obtain the desired final concentrations of the different compounds. Evaluation of the capacity factors of the solutes was performed adding to the sample vial 15 μL of anthracene standard stock solution. The aqueous phase of the microemulsion system was constituted by 10 mM sodium borate and was prepared adding an accurately weighed amount of sodium borate to a volumetric flask. The resultant pH was 9.2.

Microemulsions were prepared by mixing in a beaker the aqueous phase with a proper amount of cosurfactant (*n*-butanol), then adding an accurately weighed amount of surfactant (SDS) and, finally, an appropriate amount of oil (*n*-heptane). The addition of each component was made only after reaching a complete dissolution of the previously mixed compounds and all mixtures were continuously stirred until optically transparent microemulsion systems were obtained. All microemulsions were prepared on a w/w basis. The microemulsions were stable at room temperature for 1 month; after this period they were prepared again. During the optimization step, 20 mL and during the validation step, 500 mL of each tested microemulsion were prepared.

The percentage of the microemulsion components considered was 88.0–93.9% for the aqueous phase, 0.1–2.0% for the oil phase and 6.0–10.0% for the mixture S/CoS in a 1:2 ratio; the optimized microemulsion run buffer consisted of 90.0% 10 mM borate buffer, 2.0% *n*-heptane, and 8.0% SDS/*n*-butanol in 1:2 ratio. The optical transparency of the microemulsions was verified by measuring their transmittance at 550 nm [7]. Before use all microemulsions were filtered through 0.45 μm cellulose acetate syringe filters and further stirred. The solution for tablet assay was prepared as already described by the authors in [19], obtaining a final test concentration for KT of about 2 mg/mL.

2.3 Apparatus and operating conditions

A Simplicity 185 system (Millipore, Billerica, MA, USA) was employed to purify water previously treated by electrodeionization using an Elix system (Millipore). A 300 Ultrasonik ultrasonic bath (Ney Company, Bloomfield, USA) was used to sonicate solutions and a Metrohm 691 pH Meter (Metrohm, Herisau, Switzerland) was employed to measure pH. Microemulsions were stirred with a multiple magnetic stirrer Multipoint HP15 (Variomag, Daytona Beach, FL, USA) and their transmittance was measured with a UV-1601 spectrophotometer (Shimadzu Italy, Milan, Italy). An Agilent Technologies ^{3D}CE system (Agilent Technologies, Waldbronn, Germany) equipped with an on-column UV-visible diode-array detector (DAD) and an air thermostatting system was employed for all separations. The vial carousel was at room temperature. Data acquisition and signal processing were performed using ^{3D}CE ChemStation software (Rev. A.09.01, Agilent Technologies).

The fused-silica capillaries (50 μm ID, 375 μm OD) were purchased from Composite Metal Services (Hallow, UK) and had a total and effective length of 33.0 and 8.5 cm, respectively. The detection window was built-in by burn-

ing off the polyimide coating on the capillary. The detection wavelength was 323 nm, which is near (or actually corresponded to) the maximum wavelength for all the analytes and the internal standard. Sample introduction was performed hydrodynamically (50 mbar for 5 s) from the outlet *via* short-end injection, with the anode at the outlet and the cathode at the inlet side. The separation run was carried out in reverse polarity mode, at −17 kV and 17°C. Before use, a new capillary was flushed with 1 M NaOH and water for 5 min each. At the beginning of each day, the capillary was rinsed with 0.1 M NaOH for 2 min and then with water for 2 min. Between the runs the capillary was flushed as follows: 1 min with water, 2 min with methanol, 1 min with 1 M sodium hydroxide, 1 min with 0.1 M sodium hydroxide, 1 min with water and finally 4 min with run buffer. Prior to each sequence, two blank injections were performed to stabilize the capillary wall surface and to allow the buffer and the sample solutions to reach a constant temperature on the autosampler tray.

During optimization of the microemulsion system and of the instrumental parameters voltage and temperature, the electrophoretic experiments were run in a randomized order with KT concentration of 2 mg/mL and HK, KK and DK concentrations of 20 μg/mL.

2.4 Calibration curves

In order to obtain calibration curves, each analyte/internal standard peak area ratio was plotted *versus* each analyte/internal standard concentration ratio, using TL as internal standard. The regression curves for drug substances and drug product were evaluated for KT at around 40–120% of the test concentration of 2 mg/mL. The considered ranges for HK, KK and DK were from the respective LOQ values to 1% w/w with respect to the principal component: HK, 2.0–20.0 μg/mL (0.10–1.00% w/w); KK, 1.0–20.0 μg/mL (0.05–1.00% w/w); DK 1.6–20.0 μg/mL (0.08–1.00% w/w). TL concentration was 0.5 mg/mL and was kept constant throughout all the experiments.

As regards drug substance, the different concentrations of each analyte, together with the internal standard, were prepared by adding the proper volumes of the standard stock solutions to different vials and diluting to 500 μL with 10 mM sodium borate. With regard to drug product, separate weightings of synthetic mixtures of the tablet components were used.

2.5 Calculations and software

Resolution values R were calculated according to the formula

$$R = 1.18(t_{RA} - t_{RB}/W_{1/2A} + W_{1/2B})$$

where t_{RA} and t_{RB} are the migration times and $w_{1/2A}$ and $w_{1/2B}$ are the peak widths at half height of adjacent peak pairs, respectively [42].

Experimental design was generated and statistical analysis of experimental data was performed using NEMROD-W software package (LPRAI Sarl, Marseille, France) [43].

3 Results and discussion

The method development for the determination of KT and its impurities consisted of an optimization phase of the MEEKC method, followed by a validation step. For quantitative purpose, tolmetin was chosen as internal standard; thus the separation involved five analytes (KT, TL, HK, KK and DK).

In general, the requirements for use of an analytical method in a routine pharmaceutical analysis include a relatively rapid separation [39]. In order to fulfil this requirement, the short-end injection method was used operating in reversed-polarity mode. The migration order of the analytes was KT, TL, HK, KK and DK.

3.1 Microemulsion system optimization by mixture design

Normally, stable microemulsions can be found using the titration method which involves a large number of test mixtures to be prepared and analyzed in order to cover, as much as possible, the different possible combinations. A more favorable solution is to set up experiments according to a statistical experimental design [26, 29] that in this case involves mixture experiments. In general, experimental design can be used to obtain a good description and prediction of the considered problem. Through the study of a map (*i.e.* response surface), faithfully representing the problem, it is possible to identify the conditions yielding the best results.

Mixture experiments are a special class of response surface experiments in which the product under investigation is made up of several components or ingredients. The quantities of components, measured in weights, volumes or some other units, add up to a common total, and the mixture ingredients cannot be varied independently. The response (the quality or performance of the product based on some criterion) is a function of the proportions of the different ingredients in the mixture, not their absolute amount. In contrast, in an experimental design for process factors, the response depends on the amount of each factor [28–30].

As for the one-variable-at-a-time approach, the first step of a multivariate optimisation concerns the choice of the influential factors and responses. The experiments are then planned in order to homogeneously cover the experimental space for which limits are defined by the researcher [29].

Based on previous results [1, 2], sodium borate 10 mM (pH 9.2) was chosen as aqueous phase, SDS was chosen as surfactant, and *n*-butanol was used as cosurfactant, while *n*-heptane was selected as oil phase. Surfactant was blended with cosurfactant in a fixed weight ratio of 1:2; thus, the mixture optimization involved a three-component system.

Constraints of the components were imposed according to literature [1, 2]: X_1, aqueous phase (*W*), ranged from a lower to upper bound of 88.0 and 93.9%, respectively; X_2, oil phase (*O*), from 0.1 to 2.0%; and X_3, S/CoS in a 1:2 ratio from 6.0 to 10.0%. The prepared mixtures were then tested as BGE in MEEKC.

Initially the measured responses were the resolution values between adjacent peak pairs, *i.e.* R_1 (KT/TL), R_2 (TL/HK), R_3 (HK/KK), R_4 (KK/DK), and analysis time (*t*), measured as migration time of the last peak (DK migration time), which were to be maximized and minimized, respectively.

The composition of the microemulsions was correlated with the measured properties by the following Scheffé special cubic model [29, 30]:

$$y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_2 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 + \varepsilon$$

where *y* represents the response, the variable x_1 the aqueous phase, x_2 the oil phase and x_3 the S/CoS. The model coefficients β_i can be interpreted as the expected value of the response when 100% of the mixture is composed of ingredient *i* and all other components have proportion zero [29]. If constrained design with lower and/or upper bounds is present, as in this case, pseudo components, that are combinations of the original components, are calculated. Pseudocomponents rescale the constrained data area so that the minimum allowable amount of each component is zero. This transformation allows a more accurate estimation of model coefficients to be obtained with respect to that obtainable using the original component system [29, 30].

A 13-run experimental plan was carried out in order to estimate the coefficients of the postulated model which, for an optimization problem such as this, should be predictive in order to simulate the behaviour of the studied properties inside the experimental domain of interest. Transmittance was measured for all prepared microemulsions in order to check their stability and homogeneity [7] and it was around 100%.

Table 2 shows the experimental plan in pseudo components together with the measured responses. A visual inspection of the obtained results pointed out that good separations of all considered analytes ($R > 1.5$) were obtained with several microemulsions tested. In addition, the statistical treatment of the responses showed that the special cubic regression model was significant and valid for responses *t* and R_1 (KT/TL), while for the other responses (R_2, R_3, R_4) the model was found not valid or not significant. However, since no problem was observed for these responses, which showed resolution values above 3, it was possible to not consider them for the statistical treatment.

Table 2. 13-Run mixture experimental plan and responses

Exp. no.	*W* (% w/w)	*O* (% w/w)	S/CoS (% w/w)	*t* (min)	R_1	R_2	R_3	R_4
1	93.90	0.10	6.00	2.40	1.10	12.84	4.91	7.23
2	92.00	2.00	6.00	2.73	1.35	11.99	4.12	10.23
3	89.90	0.10	10.00	4.68	4.49	14.47	5.04	11.08
4	88.00	2.00	10.00	4.24	4.04	6.06	3.26	9.52
5	92.95	1.05	6.00	2.54	1.15	12.98	4.89	9.46
6	91.90	0.10	8.00	3.54	2.49	15.80	4.66	10.25
7	90.00	2.00	8.00	3.58	2.81	10.93	3.81	11.57
8	88.95	1.05	10.00	4.52	4.23	7.79	3.68	10.22
9	90.95	1.05	8.00	3.70	2.84	9.26	3.52	9.36
10	92.43	0.58	7.00	3.15	1.85	14.81	4.21	10.27
11	91.47	1.53	7.00	3.12	1.98	14.51	4.57	11.65
12	90.43	0.58	9.00	4.10	3.52	8.56	3.17	10.08
13	89.48	1.53	9.00	4.01	3.33	13.11	4.72	10.57

In particular, the significance of the calculated empirical model was assessed by means of the analysis of variance (ANOVA) [29, 44], while the validity of the model was assessed with residual analysis, using points no. 10, 11, 12 and 13 (Table 2) as test points. A comparison between the obtained responses and the predicted responses from the model was made and no statistically significant difference was observed [29]. The indication of acceptability from the test points was also confirmed by checking the lack-of-fit of the model. ANOVA for responses *t* and R_1 is reported in Table 3.

Table 3. ANOVA for response *t* and R_1

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F* ratio
t				
Regression	6.5980	6	1.0997	195.32[a)]
Residuals	0.0507	9	0.0056	
Lack-of-fit	0.0177	6	0.0029	0.27[b)]
Pure error	0.0330	3	0.0110	
Total	6.6487	15		
R_1				
Regression	16.4528	6	2.7421	311.89[c)]
Residuals	0.0791	9	0.0088	
Lack-of-fit	0.0581	6	0.0097	1.38[d)]
Pure error	0.0210	3	0.0070	
Total	16.5320	15		

a) $195.32 > F_{crit} = 3.37$ (with 6 and 9 degrees of freedom and $\alpha = 0.05$).
b) $0.27 < F_{crit} = 8.94$ (with 6 and 3 degrees of freedom and $\alpha = 0.05$).
c) $311.89 > F_{crit} = 3.37$ (with 6 and 9 degrees of freedom and $\alpha = 0.05$).
d) $1.38 < F_{crit} = 8.94$ (with 6 and 3 degrees of freedom and $\alpha = 0.05$).

The calculated models in pseudocomponents were the following for *t* and R_1, respectively:

$$t = 2.394x'_1 + 2.310x'_2 + 5.621x'_3 + 1.541x'_1x'_2 + 0.404x'_1x'_3 - 1.482x'_2x'_3 + 1.819x'_1x'_2x'_3$$

$$R_1 = 1.083x'_1 + 3.659x'_2 + 6.548x'_3 - 2.527x'_1x'_2 - 1.573x'_1x'_3 - 7.455x'_2x'_3 + 7.807x'_1x'_2x'_3$$

The use of the models in a predictive way to reveal the optimal microemulsion was supported by the contour plots (Fig. 1). In these plots, lines with the same predicted values of the considered response (isoresponse lines) are reported.

Figure 1a reports the contour plot for *t* in the region defined by the pseudo components of aqueous phase, oil and S/CoS. Each corner corresponds to the points representing the upper bounds of each substance, thus by moving away from each one of them the percentage of the relative component decreases. For example, the corner indicated with X_1 corresponds to the upper bound of the experimental domain defined for the aqueous phase and to the lower level of the other ingredients. Starting from this principle, from the figure it is possible to see that by increasing the percentage of buffer, the response analysis time decreases, while an opposite effect is observed by increasing the percentage of S/CoS. The oil effect is not important at low values of S/CoS, but it becomes important at high values of S/CoS. In particular, for a high percentage of S/CoS, high percentages of oil are requested to decrease analysis time.

As regards response R_1, the obtained isoresponse lines are reported in Fig. 1b. In this case it appears that the important components are the percentages of buffer and S/CoS. Resolution increases using high S/CoS and decreasing buffer percentage. The oil effect is related to the percentage of S/CoS, demonstrating the presence of an interaction between these two components. In fact, with a low percentage of S/CoS it is preferable to use a high percentage of oil, while with a high percentage of S/CoS a low percentage of oil led to better resolution values.

From the above results it clearly appears that the conditions required to optimize *t* are in conflict with the values needed to optimise R_1. A simple way to resolve the problem is to use Derringer's desirability function (*D*) [14, 15, 19, 29]. This function is a measure of overall quality and provides convenient means to compare several responses and to select the optimum with the most desirable properties. Starting from its bidimensional or 3-D graphical representation, it is possible to find the best conditions to simultaneously optimize different responses [29]. The measured responses are transformed to a dimensionless partial desirability (*d*) scale that ranges between $d = 0$, for a completely undesirable response, and $d = 1$ for a fully desirable response. Then the overall quality *D* is calculated by way of the geometric mean:

$$D = (d_1 x d_2 x \ldots d_m)^{1/m}.$$

This function combines the desirability values established for the different responses [29, 45]. Depending on the importance attributed to a response, the individual d_i functions can be weighed and the total *D* function assumes the form:

$$D = (d_1^{w_1} x\ d_2^{w_2} x\ \ldots\ d_m^{w_m})^{1/(w_1 + w_2 + \ldots w_m)}$$

The value of *D* is the highest under conditions where the different criteria are simultaneously satisfied [45, 46].

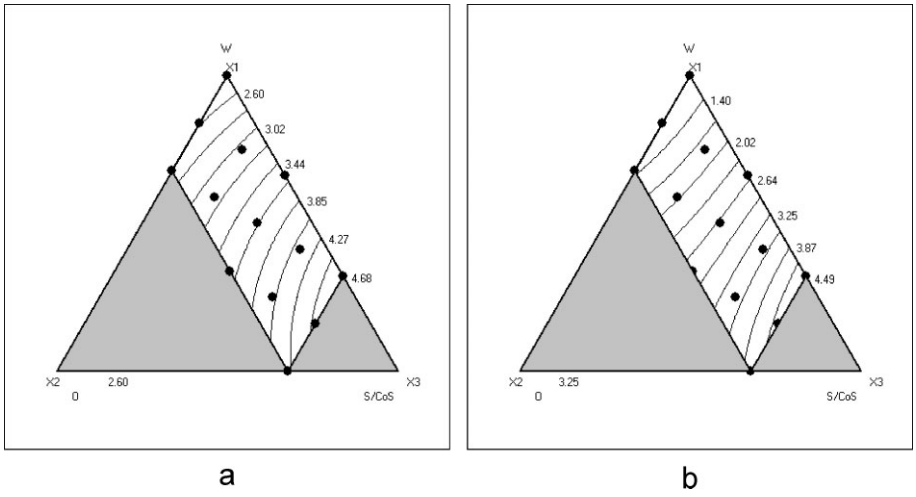


Figure 1. Contour plots. (a) Response *t*; (b) response R_1. Experiments are located by circles. X_1, aqueous phase (*W*); X_2, oil phase (*O*); X_3, surfactant/cosurfactant (S/CoS).

In this study the partial desirability functions d_i and *D* were calculated by means of the NEMROD-W software [43] and it was possible to obtain the best compromise between analysis time (to be minimized) and resolution (to be maximized). In particular, for the response analysis time a desired value of 3 min and a maximum of 4 min were set, while for the response R_1 a minimum and a desired value corresponding to 2 was set.

The desired requirements were fulfilled by two microemulsions: microemulsion A that was predicted and corresponded to 92.4% buffer, 0.1% oil and 7.5% S/CoS, and microemulsion B that corresponded to no. 7 of the experimental plan, and was composed of 90.0% buffer, 2.0% oil and 8.0% S/CoS. The special cubic model resulted to be valid in a predictive way [29, 35] with respect to both microemulsions, for both responses.

3.2 Optimization of process factors by experimental design

After optimization of the microemulsion system, with the aim of further reducing analysis time maintaining a baseline resolution between KT and tolmetin, an experimental design for process factors [29] was carried out in the experimental domain defined by the instrumental parameters temperature and voltage.

The considered factors X_1, voltage (*V*) and X_2, temperature (*T*), were studied in the range of 14–22 kV and 17–23°C, respectively. The considered responses were the critical resolution R_1 and analysis time *t*. In order to find the conditions able to minimize analysis time maintaining a baseline resolution among the analytes, a response surface study was carried out.

The experiments were planned assuming a second-order polynomial relationship between response and factors:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \varepsilon$$

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 five-level central composite design (CCD) [29, 47] was used to find the coefficients of the postulated second-order polynomial function. This design allowed the response surface to be modelled with a number of experiments equal to $2^k + 2k + n$, where *k* is the number of variables and *n* is the number of extra points at the centre of the design. These replicates give a measure of experimental error, and thus, the significance and validity of the model can be assessed.

The experimental plan together with the obtained responses for microemulsions A and B is reported in Table 4.

The hypothesized model was found valid and significant for both responses obtained with both microemulsions. However, the results obtained with microemulsion B were better. Good resolutions were obtained with short analysis time and microemulsion B was chosen as BGE for the MEEKC analysis. In particular, for this microemulsion desirability function made it possible to find a point characterized by a value of temperature and voltage equal to 17°C and 17 kV, respectively, where the predicted values of the responses were 2.26 for resolution and 2.73 for analysis time.

Table 4. CCD experimental plan and responses (t, R_1) for microemulsions A and B

Exp. no.	V (kV)	T (°C)	t A (min)	R_1 A	t B (min)	R_1 B
1	15	18	3.12	2.17	3.34	2.41
2	21	18	1.81	1.84	1.78	1.91
3	15	22	2.34	1.86	3.03	2.37
4	21	22	1.56	1.68	1.63	1.83
5	14	20	3.20	2.17	3.72	2.73
6	22	20	1.48	1.65	1.53	1.77
7	18	17	2.46	2.06	2.49	2.22
8	18	23	2.03	1.90	2.15	2.05
9	18	20	2.31	1.89	2.33	2.09
10	18	20	2.30	1.91	2.34	2.06
11	18	20	2.20	1.96	2.32	2.11

The desirability graph, shown in Fig. 2, pointed out that only few combinations led to acceptable values of desirability, while in a large zone of the investigated experimental domain desirability was 0. In particular, the voltage value appeared to be critical, while temperature seemed to be less important. In fact, for a value of voltage around 17 kV, all the temperature values inside the investigated experimental domain led to acceptable values of desirability.

By applying the predicted conditions, separation of the analytes was obtained in about 3 min and the resolutions were greater than 2 (Fig. 3). At this point the model was validated in a provisional way, at a probability level of 95%, applying the formula:

$$\hat{y}_u \pm t_{\alpha/2,\nu}\left(\frac{1}{m} + d_u\right)^{1/2} s$$

in which $\hat{y}_u$ is the predicted value, m is the number of experimental replicates, d_u is the variance function in the predicted point and s is the calculated SD [29]. The found experimental value was inside the calculated interval.

3.3 MEEKC capacity factors

The separation window was defined by using a double internal marker approach. The EOF marker was acetone, which is not retained by the separation carrier, while the microemulsion marker was anthracene, which is exclusively transported by the separation carrier and is not transported by the mobile phase [48].

The capacity factors of the neutral solutes, indicating the extent of solute association with microemulsion droplets, were calculated according to the well known formula, valid for neutral and weakly ionised solutes [49]:

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

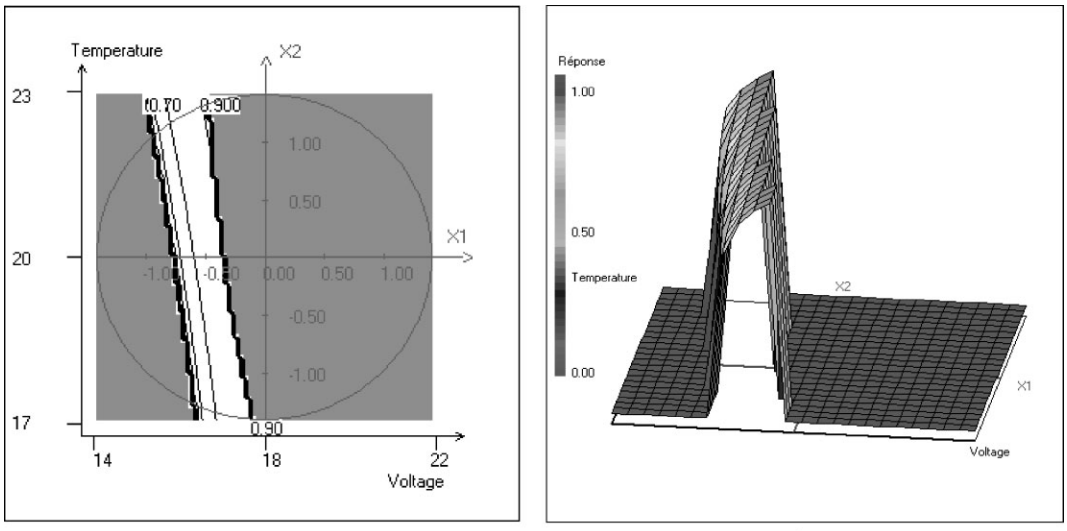


Figure 2. Desirability function (a) 2-D and (b) 3-D plot obtained by plotting voltage (V) *versus* temperature (T). Microemulsion used: 90.0% 10 mM borate, 2.0% *n*-heptane, 8.0% SDS/*n*-butanol in a ratio of 1:2.

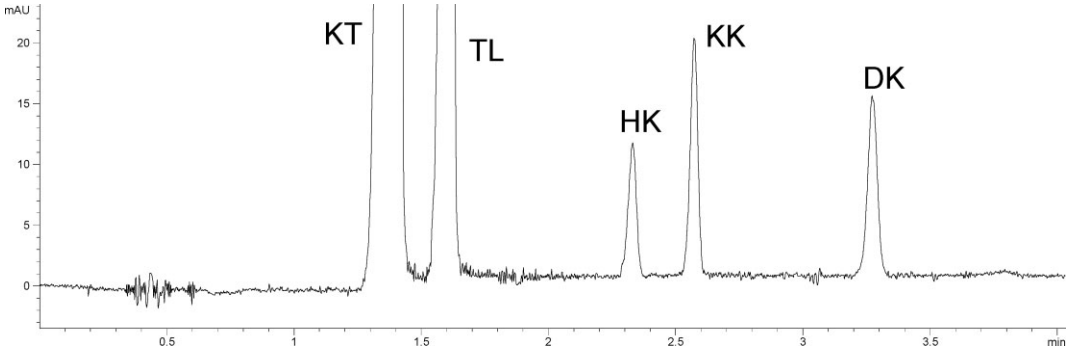


Figure 3. Electropherogram of KT and related substances referring to optimal conditions: voltage, −17 kV; temperature, 17°C. Microemulsion: 90.0% 10 mM borate, 2.0% *n*-heptane, 8.0% SDS/*n*-butanol in a ratio of 1:2. Short-end injection: 50 mbar, 5 s. Detection wavelength: 323 nm.

where t_m is the migration time of the solute, t_0 is the migration time of the unretained solute moving at the EOF rate and t_{me} is the microemulsion droplet migration time [49].

In the case of KT and tolmetine, these are solutes which are in equilibrium with the deprotonated species; thus, the migration behavior is mostly described in electrophoretic rather than chromatographic terms. In any case, the corrected capacity factor (k'_{corr}) can be calculated by means of the following formula [49]:

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

where t_m and t'_m are the migration times of the solute in MEEKC and CZE conditions, respectively. This formula is valid if the electroosmotic mobility values in MEEKC and CZE modes are identical. In this case, the electroosmotic mobility changed by about 10% between MEEKC and CZE conditions.

The detection wavelength was set at 250 nm and the following capacity factors were calculated (n = 4, $\alpha/2$ = 0.025): KT, 1.50 ± 0.09; TL, 2.13 ± 0.13; HK, 5.81 ± 0.31; KK, 8.39 ± 0.49; DK, 27.68 ± 1.97. The low values for KT and TL are justified considering that at pH 9.2 both these compounds are in anionic form and their affinity for the negatively charged microemulsion droplets is low. On the other hand, for the impurities, the lipophilicity and thus the affinity for the microemulsion droplets increases in the order HK < KK < DK.

3.4 Validation

The MEEKC method was validated applying the optimized experimental conditions (BGE: 90.0% 10 mM sodium borate, 2.0% *n*-heptane, 8.0% SDS/*n*-butanol in 1:2 ratio; temperature: 17°C; voltage: −17 kV), following the ICH guidelines [41] and using reference standards of KT and impurities. Unless otherwise stated, validation parameters were evaluated using a test mixture of 2 mg/mL KT and 11 μg/mL HK, KK and DK (0.55% w/w, *i.e.* about the mid point within their linearity range). For quantitative application, the internal standard method was necessary and TL (0.5 mg/mL) was selected as internal standard. This is an acidic molecule which shows a high absorbance at detection wavelength 323 nm and a lower analysis time than KT impurities.

3.4.1 Selectivity

Selectivity of the method was verified by analyzing mixtures of the analytes separately spiked with each of the compounds, and additionally by analysing standard solutions of each single analyte. The online recorded UV spectra (DAD) further confirmed the identity of the peaks. The peaks were baseline separated as demonstrated by the resolution values measured (n = 4, $\alpha/2$ = 0.025): R_1 (KT/TL), 2.63 ± 0.30; R_2 (TL/HK), 17.57 ± 0.90; R_3 (HK/KK), 5.22 ± 0.20; R_4 (KK/DK), 10.68 ± 0.90. Finally, no disturbance in the electropherograms was observed due to the presence of excipients.

3.4.2 Robustness

In CE there are a large number of factors which are potentially critical for method robustness [50–52]. In our case, we dealt with two kinds of factors: mixture and process variables. The first type of factors included aqueous phase, S/CoS and oil phase, while the second type included temperature, voltage and buffer concentration. This last factor was the only one not considered in the optimisation phase, as generally MEEKC is performed using low-ionic strength borate or phosphate

Table 5. Six-factor[a] eleven-run D-optimal experimental plan and responses[b] for robustness testing

Exp. no.	*W* (% w/w)	S/CoS (% w/w)	*O* (% w/w)	*T* (°C)	*V* (kV)	Conc. (mM)	*t* (min)	R_1
2	89.93	8.07	2.00	18	16	9	3.75	3.11
6	90.07	7.93	2.00	16	16	11	3.79	2.83
9	90.07	7.93	2.00	18	18	11	3.06	3.18
13	89.93	8.00	2.07	16	18	9	3.04	3.10
14	89.93	8.00	2.07	18	18	11	3.42	3.25
17	90.07	8.00	1.93	18	16	9	3.64	3.09
21	90.00	8.07	1.93	16	16	11	3.63	3.22
23	90.00	8.07	1.93	16	18	9	3.78	3.20
27	90.00	7.93	2.07	18	16	9	3.36	2.61
31	90.00	8.00	2.00	16	16	11	4.02	3.48
34	90.00	8.00	2.00	18	18	11	2.93	3.09

a) Factors: *W*, aqueous phase; S/CoS, surfactant/cosurfactant; *O*, oil phase; *T*, temperature; *V*, voltage; Conc., buffer concentration.
b) Responses: *t*, analysis time; R_1, KT/TL resolution.

buffers, which generate relatively low currents and a reasonably fast EOF [1]. Thus, in a first step it did not seemed necessary to optimise this factor, but we decided only to check by robustness testing if a small variation in buffer concentration would affect the analysis performances. The selected responses were the same considered for optimization, *i.e.* analysis time (*t*) and the critical resolution between KT and TL (R_1).

Symmetrical values around the optimized level were selected for factor levels, which were assumed to reflect the variations which could be encountered in different laboratories. The experimental domain tested was: X_1, aqueous phase (*W*), 89.9%–90.1%; X_2, S/CoS, 79.0–81.0%; X_3, oil phase (*O*), 1.9–2.1%; X_4, temperature (*T*), 16–18°C; X_5, voltage (*V*), 16–18 kV; X_6, buffer concentration (Conc.), 9–11 mM.

The experimental plan was made up of eleven experiments and was obtained applying the D-optimal algorithm [29, 47] to a 40-run experimental matrix [29] able to simultaneously evaluate the effect on the response of mixture and process variables [53]. The D-optimal algorithm makes it possible to obtain the best compromise between quality of information and number of experiments [29, 47]. The experimental plan together with the obtained responses is shown in Table 5.

No statistically significant variation was pointed out in the observed responses. In particular, ANOVA [29, 47] showed that the variation of the responses was not explained by factor variation, thus indicating method robustness.

3.4.3 Migration time and peak area precision

In order to determine the within-day precision of migration times and corrected peak areas, replicate injections ($n = 6$) of the standard mixture were carried out (Table 6). The between-day precision was evaluated over a 3-day period by performing six successive injections each day. The RSD values obtained were satisfactory and were in the same order of magnitude for within-day and between-day precision, apart from DK corrected peak area ratio for which an unusually high between-day RSD was reported. However, as shown below, this behavior did not interfere with the capacity of the method for an accurate and precise determination of DK.

Table 6. Within-day and between-day precision data for analysis time (DK migration time) and corrected peak area ratios for a test mixture of 2 mg/mL KT, 11 μg/mL HK, KK, DK and 0.5 mg/mL TL (internal standard)

	RSD (%, $n = 6$)			RSD (% $n = 18$)
	Day 1	Day 2	Day 3	Days 1–3
Corrected peak area ratios[a]				
A_{KT}/A_{TL}	0.8	0.3	0.6	0.9
A_{HK}/A_{TL}	3.8	2.0	2.4	3.6
A_{KK}/A_{TL}	2.9	2.9	1.4	4.1
A_{DK}/A_{TL}	2.5	4.0	2.5	9.0
Analysis time	3.7	1.0	1.7	3.0

a) A_{KT}, KT corrected area; A_{HK}, HK corrected area; A_{KK}, KK corrected area; A_{DK}, DK corrected area; A_{TL}, TL corrected area.

From the data obtained it clearly appeared that the internal standard method was essential to circumvent injection variation observed applying hydrodynamic injection.

3.4.4 LODs and LOQs

LOD and LOQ of the three impurities were estimated as three times and ten times the signal-to-noise ratio (S/N), respectively. S/N was calculated by means of Agilent Technologies ChemStation software and the limits were determined by injecting mixture solutions of different concentrations. Validation of the LOQ was then performed for both drug substance and drug product by means of replicating eight analyses at LOQ concentrations. The obtained LOD and LOQ values and the corrected area ratio RSD values are reported in Table 7.

Table 7. LOD and LOQ concentration values and validation of LOQ values for ketorolac impurities

Analyte	LOD (μg/mL)	LOD, (% w/w)	LOQ (μg/mL)	LOQ (% w/w)	RSD (%, $n = 8$, LOQ) Drug substance	RSD (%, $n = 8$, LOQ) Drug product
HK	1.0	0.050	2.0	0.100	6.2	4.8
KK	0.7	0.035	1.0	0.050	6.6	7.3
DK	0.8	0.040	1.6	0.080	7.2	5.6

3.4.5 Linearity

The linearity of the method was evaluated for both drug substance and drug product. In this last case, a placebo tablet solution was used to obtain the calibration curves. The curves consisted of five data points and two replicate injections at each concentration level. The data obtained are presented in Table 8.

3.4.6 Accuracy and precision

Accuracy and precision of the MEEKC method were evaluated by means of analysing three concentration levels for each analyte with three replicates in order to cover the linearity range. In the case of drug substances, the method was applied to standards of known purities, while in the case of drug product the analyses were performed on synthetic mixtures of the drug product excipients to which standards had been added. Values of recovery together with the confidence interval and values of RSD obtained are shown in Table 9.

3.4.7 System suitability test limits

System suitability test (SST) limits can be determined when the method is considered robust for its quantitative assay [54]. In this case, SST limits were derived from robustness test as recommended, in fact in this test the most extreme variations in the factors that still are probable under acceptable conditions are examined [54]. Thus, the lowest and highest response measured defined the range accepted for the method performances, *i.e.* $2.93 < t < 4.02$ and $2.61 < R_1 < 3.48$. If an experiment run in the optimised conditions presents values of the responses included in this interval, this assures that validity of the analytical procedure is maintained.

Table 8. Linearity data[a)] obtained for KT and KT-related substances ($n = 5$, $k = 2$)

Analyte	Range (mg/mL)	a	s_a	b	s_b	SE	R^2	R^2_{CV}
Drug substance								
KT	0.8–2.4	0.8352	0.0103	−0.0518	0.0351	0.0117	0.9988	0.9981
HK	0.002–0.020	1.1625	0.0235	−0.0009	0.0006	0.0003	0.9967	0.9949
KK	0.001–0.020	2.0198	0.0335	−0.0010	0.0009	0.0005	0.9978	0.9964
DK	0.0016–0.020	1.4467	0.0324	−0.0007	0.0008	0.0004	0.9960	0.9935
Drug product								
KT	0.8–2.4	0.7826	0.0068	0.0961	0.0230	0.0077	0.9994	0.9990
HK	0.002–0.020	1.0192	0.0227	0.0013	0.0006	0.0003	0.9961	0.9940
KK	0.001–0.020	1.6101	0.0221	0.0006	0.0006	0.0003	0.9985	0.9975
DK	0.0016–0.020	1.2181	0.0235	−0.0005	0.0006	0.0003	0.9970	0.9958

a) Regression equation, $y = ax + b$; s_a, SD for the slope; s_b, SD for the intercept.

Table 9. Accuracy and precision data for the assay of KT and related impurities for drug substances and for drug product (n = 3, $\alpha/2$ = 0.025)

Analyte	Conc. level (mg/mL)	Drug substance		Drug product	
		Accuracy (recovery, %)	Repeatability (RSD, %)	Accuracy (recovery, %)	Repeatability (RSD, %)
KT	0.900	102.5 ± 1.6	0.6	101.7 ± 0.6%	0.2
	2.000	98.4 ± 0.4	0.7	99.7 ± 0.9%	0.4
	2.300	100.7 ± 1.4	0.6	98.7 ± 1.4%	0.6
HK	0.003	106.6 ± 12.4	4.7	98.8 ± 9.3%	3.8
	0.011	96.0 ± 13.2	5.5	104.0 ± 7.9%	3.1
	0.019	98.4 ± 2.9	1.2	102.2 ± 6.0%	2.3
KK	0.003	102.3 ± 13.1	5.2	97.9 ± 13.6%	5.6
	0.011	97.4 ± 6.4	2.7	101.5 ± 2.3%	0.9
	0.019	103.1 ± 8.1	3.2	100.2 ± 4.0%	1.6
DK	0.003	95.6 ± 10.0	4.2	95.5 ± 10.2%	4.3
	0.011	94.6 ± 7.3	3.1	102.6 ± 13.3%	5.2
	0.019	99.9 ± 4.6	1.8	98.0 ± 9.1%	3.7

3.5 Applications

The developed method was applied to the analysis of Lixidol-coated tablets, which are labelled to contain 10 mg KT tromethamine each. Four replicates were carried out and the results were in agreement with the claimed content (n = 4, $\alpha/2$ = 0.025): recovery 97.1 ± 0.6%, RSD 0.4%. The electropherogram of Lixidol is reported in Fig. 4. Among the impurities, only KK was detected at concentration levels near the respective LOD (0.7 μg/mL). The identity of the impurity peak was further confirmed by means of spiking with a proper amount of KK standard solution. From the obtained electropherogram, it appears that a reduction of migration time of the impurity KK is present, when compared to the analysis under standard conditions (Fig. 3). The spike of the other impurities in the real sample showed a migration time reduced also for these analytes; however, resolution values were still good and suitable for the quantitative analysis. This phenomenon was probably due to the partial break down of the microemulsion system caused by the presence of methanol [55] in the sample. Unfortunately, it was not possible to reduce the injected volume since it would have reduced the amount of the impurities, making them not quantifiable.

3.6 Comparison of MEKC, MEEKC and CEC methods

In our laboratory, the separation of KT and its related substances has been studied in three different CE systems: MEKC [19], CEC [38] and MEEKC, thus allowing a comparison of the characteristics of these three operative modes in a particular case of pharmaceutical quality control.

As regards the preparation of the stationary and pseudostationary phases used, in CEC packing of the capillaries requires a certain training of the personnel. MEKC is the most practical technique, as it needs only the addition of the surfactant (SDS) to the aqueous buffer. In MEEKC the composition of the BGE is more complex, involving also an oil phase (*n*-heptane) and a cosurfactant (*n*-butanol). On the other hand, the complexity of the microemulsion system in principle makes it possible to more finely tune the separation conditions than in MEKC. In this case, the order of migration of the compounds in both the MEKC and MEEKC systems was the same. Overall, the highest selectivity is observed with CEC, as it is the only method which makes it possible to easily change the retention order of the compounds by changing pH of the mobile phase.

Considering the analysis performances, MEEKC presents the lowest analysis time (about 3 min), which is about doubled and tripled for MEKC and CEC, respectively. In general, as observed previously, the microemulsion system provides much faster migrations compared to the MEKC system [56]. However, in this case the rapidity of MEEKC analysis is more immediately explained by the application of the short end injection mode. Times of conditioning were of the same order of magnitude in MEKC (6 min) and MEEKC (10 min). In particular, the higher conditioning time for MEEKC is explained by the need to add methanol to the washing solvents in order to

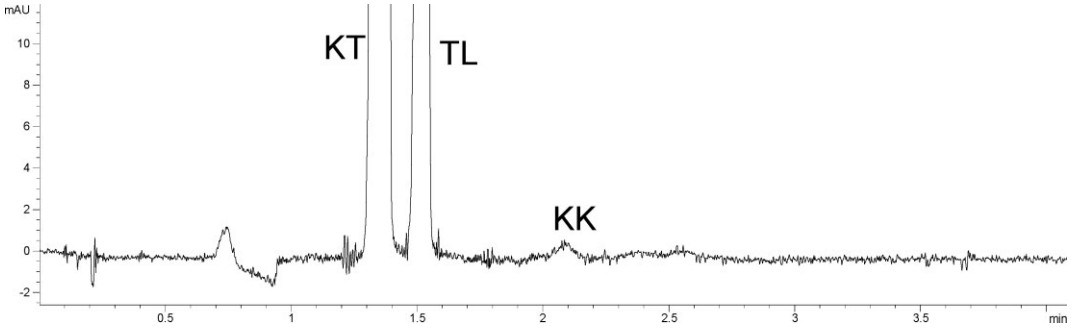


Figure 4. Electropherogram of a real sample of Lixidol-coated tablets referring to optimal conditions. For experimental conditions, see Fig. 3.

obtain a good repeatability. In CEC the main time-consuming step is the conditioning at the beginning of each day (about 1 h): first applying pressure and then both pressure and voltage until a stable current and baseline signal are detected.

Finally, validation parameters are satisfactory for all the considered methods, showing the potential of each of them in pharmaceutical quality control. LOD and LOQ for the impurities are similar for the three methods in terms of absolute values of concentration. However, with CEC it is not possible to select a very high test concentration for the main component due to a consequent lack of efficiency of KT peak. Thus, in CEC the test concentration of KT is 0.1 mg/mL, while in the EKC methods it is 2 mg/mL. This means that the EKC methods present better performances in terms of percentages with respect to the main component. However, as previously pointed out [38], the volatile mobile phase used in CEC (formate buffer) could make it possible to increase sensitivity by using a MS detector.

4 Concluding remarks

In this work, a method based on MEEKC was developed for analyzing KT and its three related substances in bulk drug and coated tablets. Optimization and validation of the method were performed applying an experimental design strategy. In particular, for the first time in literature, the best microemulsion system to be used as BGE was found by means of a mixture design, considering as components the aqueous phase, the oil phase and the mixture S/CoS. The present study confirms the usefulness of chemometric techniques in developing analytical methods involving complex systems and the potential of MEEKC in pharmaceutical quality control.

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