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Analysis of ketorolac and its related impurities by capillary electrochromatography

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Abstract

Capillary electrochromatography (CEC) was employed for the assay of ketorolac (KT) and its known related impurities [1-hydroxy analog of ketorolac (HK), 1-keto analog of ketorolac (KK), ketorolac decarboxylated (DK)] in both drug substance and coated tablets. Detection was made at 323 nm and flufenamic acid was selected as internal standard. The experiments were performed in a 100 μm i.d. capillary packed with RP-18 silica particles (33.0, 24.5, 23.0 cm total, effective and packed lengths, respectively). The composition of the mobile phase was optimised by changing pH of the buffer and acetonitrile (ACN) content and by addition of other organic modifiers (methanol, ethanol, isopropanol, *n*-propanol) in order to evaluate the effect of these factors on the method performance (efficiency, retention and resolution). The optimum mobile phase consisted of a mixture of 50 mM ammonium formate buffer pH 3.5–water–acetonitrile (10:20:70, v/v/v), while voltage and temperature were set at 30 kV and 20 °C, respectively. Applying these conditions, all peaks were baseline resolved and the analysis was performed in less than 9 min. Selectivity, repeatability of retention time and peak area, detection and quantitation limits, linearity and range, precision and accuracy were also investigated. R.S.D. and bias values obtained for all the analytes were below 5% and sensitivity was satisfactory, thus the method was deemed suitable for pharmaceutical quality control. Applying the method to coated tablets, a recovery of 98.5 ± 0.8% and an R.S.D. of 0.5% were found.

Keywords: Electrochromatography; Pharmaceutical analysis; Ketorolac

1. Introduction

Capillary electrochromatography (CEC) is a modern hybrid technique that combines some aspects of CE, such as the capillary format and the electric field, with some others typical of HPLC, such as the separation mechanism based on the interaction of solutes and solid stationary phase. As a consequence, it is possible to achieve high speed and efficiency, characteristic of electro-driven systems, and at the same time, selectivity and sample loadability comparable to those of a packed capillary LC column. CEC can be performed in open tubes or packed structures. In the latter case, the separation capillary is filled with silica particles and a high voltage is applied to the column, generating a plug-like electroosmotic flow (EOF) from the double layers at the solid–liquid interfaces (both the capillary wall and the stationary particles) along the capillary. Electroosmosis, together with electrophoretic mobility in the case of charged analytes, is responsible of the transport of the analytes through the column. The most important advantages of EOF as a driving force are that the mobile phase velocity is independent of the particle size, and the radial flow profile is essentially flat (in contrast to the parabolic profile of the laminar flow in HPLC). Thus, longer columns packed with smaller particles can be used, with the effect of higher separation efficiency and peak capacity. Another advantage of some modes of CEC is that mass transfer between the stationary and mobile phases can also be substantially faster than in HPLC [1,2].

CEC is a versatile technique and has been used for the analysis of several compounds of different nature. In general, uncharged solutes migrate through a CEC column due to

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the flow of the eluent phase only and they generally have the same velocity and retention factor as in a corresponding chromatographic system. On the other hand, the migration of an ionic solute is influenced by both the electric field in the column and the adsorption to the stationary phase [3]. Chiral and non-chiral pharmaceuticals have been effectively analysed by CEC [4–11].

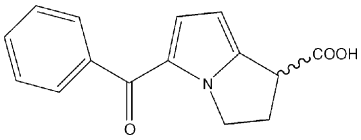
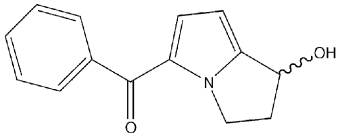
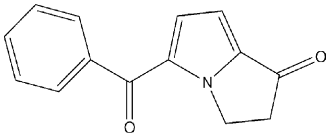
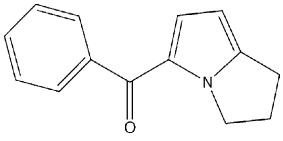
Ketorolac [KT, (±)-5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid] is a chiral nonsteroidal anti-inflammatory drug structurally related to indomethacin, possessing high efficiency and mainly employed as analgesic for the short-term treatment of moderate to severe post-operative pain. The drug is administered intramuscularly, intravenously, orally and as eye drops as the water soluble trometamol salt (1:1 compound with 2-amino-2-(hydroxymethyl)-1,3-propanediol) [12]. Its mechanism of action consists of inhibiting the enzyme cyclooxygenase, that is translated to a decrease in the metabolic products that trigger the inflammatory processes. KT may cause important gastrointestinal side effects related to cyclooxygenase inhibition, which are the major drawbacks for its use. As a high incidence of adverse effects has been reported, KT 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 [12]. The drug is administered by mouth as 2% (w/v) oral drops and 10 mg coated tablets.

Previously reported methods for KT analysis include various HPLC methods, which have been used for the quantitative determination of KT alone [13,14], KT and its hydroxylated metabolite [15], and KT enantiomers [16–20] in plasma. KT determination in biological fluids has been performed with GC–MS [21], electrospray ionization (ESI) MS–MS [22] and voltammetry [23] techniques. For the assay of KT in pharmaceutical matrices, flow injection [24], differential pulse polarography [25] and derivative adsorptive chronopotentiometry [26] methods have been developed. The United States Pharmacopeia describes an LC method as the official method for the assay of ketorolac tromethamine drug substance, injection and tablets [27].

In the pharmaceutical dosage form, as indicated by the drug substance and drug product producer (Roche, Milan, Italy), three related impurities of KT theoretically may be found, namely 1-hydroxy analog of ketorolac (HK), 1-keto analog of ketorolac (KK) and ketorolac decarboxylated (DK). The structural formulas of these compounds are reported in Table 1.

The aim of this work is to optimise a CEC separation method for the analysis of KT and its related impurities in coated tablets which may be effectively applied in pharmaceutical quality control. Currently, the vast majority of drug-related impurity determinations are performed by HPLC, but in the last few years different CE modes such as free zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) have demonstrated to be capable of the required sensitivity and precision [28]. Just a few studies have been published on the application of CEC for impurities determination [29–32]. An HPTLC method has been reported as being able to determine KT and could be extended to study the degradation kinetics of the drug in specific acid and base conditions (the expected acid product is HK) [33]. So far, only a sodium dodecyl sulfate (SDS)-based MEKC method has been developed by some of the authors for the assay of KT and the considered related

Table 1
Structural formulas of ketorolac and related substances

Compound name	Chemical structure
Ketorolac (1)	
(±)-(7-Hydroxy-6,7-dihydro-5*H*-pyrrolizin-3-yl)-phenyl-methanone (HK) (2)	
5-Benzoyl-2,3-dihydro-pyrrolizin-1-one (KK) (3)	
(6,7-Dihydro-5*H*-pyrrolizin-3-yl)-phenyl-methanone (DK) (4)	

substances [34]. It is important to continue exploring the potentialities of CEC for the routine use in the area of pharmaceutical quality control. In fact, the advantages/disadvantages of HPLC and CE in this field are well established, while those of CEC are still only partially investigated. The possibility to obtain CE benefits, such as savings in terms of solvents and sample, reduced analysis time and increased efficiency, together with HPLC typical characteristics, such as selectivity and sensitivity, makes CEC technique susceptible of a continue increase of familiarisation and training across the pharmaceutical industry. The performance of the CEC method presented was tested in terms of selectivity, repeatability of retention time and peak area, detection and quantitation limits, linearity and range, precision and accuracy.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used in this study were of analytical reagent grade with no further purification.

Ammonia solution (30%), formic acid, acetic acid and citric acid were from Carlo Erba (Milan, Italy). Acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), isopropanol (iProOH), *n*-propanol (nProOH) were obtained from BDH (Poole, UK).

Working standards of KT and its impurities (HK, KK, DK) and coated tablet excipients (cellulose microcrystalline, lactose, magnesium stearate, titanium dioxide, hydroxypropyl methyl cellulose) were furnished by Roche (Milan, Italy). Lixidol-coated tablets, each containing 10 mg of KT, were purchased from local pharmacies. Flufenamic acid (FL), used as internal standard, was from Sigma–Aldrich (St. Louis, MO, USA).

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

2.2. Solutions

Standard stock solutions of KT, HK, KK, DK, and FL (1 mg/mL) were prepared in methanol, stored at 4 °C and used within 1 week. Working standard solutions were prepared daily by adding the appropriate volume of each of the stock solutions directly in a vial and diluting to 500 μL with buffer (5 mM ammonium formate, pH 3.5) and with MeOH (up to a 50%, v/v, concentration) in order to obtain the desired final concentrations of the different compounds.

The stock buffer solution of 50 mM ammonium formate was prepared by diluting in water the appropriate volume of formic acid and adjusting to the desired pH with concentrated ammonia solution. Mobile phases used for electrochromatographic experiments were prepared daily by mixing appropriate volumes of stock buffer solutions, water and organic solvents in order to obtain a 5 mM aqueous buffer concentration.

2.3. Instrumentation

A FS 100b ultrasonic bath (Decon, Hove, UK) was employed to sonicate solutions.

A MicropH 2001 pH Meter (Crison, Barcelona, Spain) was used to measure pH.

An Agilent Technologies ^{3D}CE system (Agilent Technologies, Waldbronn, Germany) equipped with a UV-Vis diode array detection (DAD) system and an air thermostating system was used to carry out CEC experiments. The vial carousel was at room temperature. Detection was made at 195 nm during the optimisation phase in order to detect thiourea (EOF marker) and at 323 nm during validation in order to reduce the baseline noise. The ^{3D}CE Chemstation software (Rev. A.09.01, Agilent Technologies) was employed to collect and process data.

The fused silica capillaries (100 μm i.d. × 375 μm o.d.) were purchased from Composite Metal Services (Hallow, UK). Capillaries were packed by using a Series 10 HPLC pump (Perkin-Elmer, Palo Alto, CA, USA).

2.4. Electrochromatography

CEC capillaries were packed in our laboratory with LiChrospher 100 RP-18 (5 μm), of 100 Å pore size (Merck, Darmstadt, Germany). One end of the capillary was connected to a mechanical temporary frit to retain the packing material, while the other to a HPLC precolumn which was used as reservoir for the slurry and was connected to the LC pump. The slurry was prepared by adding 30 mg of stationary phase to 1 mL of methanol. The slurry and part of the capillary were ultrasonicated in a water bath. The capillary was packed pumping methanol at ∼2000 psi (1 psi = 6894.76 Pa) and an optical microscope was used to check the homogeneity of the bed structure. The slurry reservoir was removed and the capillary was flushed with double distilled water (∼3000 psi) to displace the packing solvent from the column bed. The frits were prepared sintering the C_{18} particles by using a heating coil (∼1000 °C for 6 s). The temporary frit was removed and excess packing material was eliminated by pumping double distilled water through the column. Finally, the column of the proper length was cut close to the inlet frit and the detection window was formed removing the polyimide capillary coating.

The total length of the capillary was 33.0 cm and the effective length 24.5 cm. The second frit was at 23.0 cm and was protected externally with epoxy resin. Before use, the capillary was conditioned by flushing the mobile phase for about 1 h with the CE instrument. At first, a 12 bar pressure was applied at the inlet end of the capillary, then both pressure (8 bar, both ends) and voltage (with an increase from 10 to 30 kV) until a stable current and baseline signal were detected.

The mobile phase consisted of a mixture of 50 mM ammonium formate pH 3.5–water–acetonitrile (10:20:70, v/v/v). Temperature was fixed at 20 °C. Voltage was set at 25 kV during the optimisation step and at 30 kV during validation in order to improve KT efficiency. Samples were injected through hydrodynamic mode applying high-pressure (12 bar, 12 s during optimisation step and 60 s during validation in order to obtain a sufficient sensitivity) at the anodic end of the capillary, followed by a mobile phase plug (12 bar, 12 s). During CEC runs both the inlet and outlet capillary ends were pressurized at 8 bar in order to avoid bubble formation. Under the optimised conditions a current of 5.6–6.0 μA was typically generated.

2.5. Calibration curves

In order to obtain the calibration curves, each analyte/internal standard peak area ratio was plotted versus each analyte/internal standard concentration ratio. The curves were evaluated by preparing seven different concentrations of each analyte and analysing the solutions twice.

The considered range for KT curve was 0.07–0.13 mg/mL, corresponding to 70–130% of the test concentration. The curves for the impurities were evaluated over the following concentration ranges: HK, 0.0012–0.0120 mg/mL (1.2–12.0%, w/w); KK, 0.0010–0.0100 mg/mL (1.0–10.0%, w/w); DK, 0.0013–0.0127 mg/mL (1.3–12.7%, w/w). Concentration of FL, used as internal standard, was held constant at 0.1 mg/mL throughout all the experiments.

2.6. Tablet assay

Twenty coated tablets were weighed and crushed to fine powder and the equivalent of 50 mg of KT, accurately weighed, was transferred into a 50 mL beaker. The content was diluted with about 25 mL of methanol, shaken vigorously, sonicated for 15 min and shaken again. The resulting suspension was then filtered through a dry filter into a 50 mL volumetric flask, and the volume was adjusted with methanol passed through the beaker and the filter walls. The obtained solution contained KT at a concentration of about 1 mg/mL. 50 μL of this solution were diluted in a vial up to 500 μL by adding 50 μL of internal standard stock solution, 250 μL of buffer (5 mM ammonium formate, pH 3.5) and 150 μL of methanol in order to achieve a KT final test concentration of about 0.1 mg/mL.

2.7. Calculation of chromatographic parameters

The efficiency values were measured as number of theoretical plates per meter (N), given by:

$$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^{1/2} \quad (1)$$

where t_R and $w_{1/2}$ are the retention time and the peak width at half height, respectively. Retention factor k' was given by:

$$k' = \frac{t_R - t_0}{t_0} \quad (2)$$

where t_R and t_0 are the retention times of the sample compound and of the EOF marker, respectively. Resolution values R_s were calculated according to the formula:

$$R_s = 1.18\left(\frac{t_{RA} - t_{RB}}{w_{1/2A} + w_{1/2B}}\right) \quad (3)$$

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

3. Results and discussion

A RP_{18} packed silica capillary was selected for this study based on the physico-chemical properties of KT and its impurities and our previous experience in the analysis of these compounds by SDS-based MEKC. KT is a weak acid with $pK_a = 3.49$, while the three impurities are all uncharged and their relative hydrophobicity increases in the order HK < KK < DK according to the calculation of MEKC capacity factors [34].

In order to develop the CEC method, the effects of the different composition of the mobile phase (in terms of buffer type and pH), of ACN concentration as organic modifier, and of addition of other organic solvents were examined. The optimum conditions were found on the basis of the influence of the above mentioned parameters on the performance of CEC analyses in terms of efficiency (N), retention factors (k') and resolution (R_s).

In the optimisation step, the concentration of all the analytes was fixed at 0.04 mg/mL. CEC experiments were carried out applying 25 kV and 8 bar at both ends of the capillary and injection was performed at the anodic end of the capillary by high-pressure application (12 bar, 12 s) followed by a mobile phase plug (12 bar, 12 s). Detection wavelength was set at 195 nm, which made it possible to detect thiourea peak (0.04 mg/mL), selected as EOF marker.

With the aim of obtaining preliminary information about the system, a mixture consisting of 50 mM ammonium formate buffer pH 3.0, water and ACN in the ratio of 10/10/80 (v/v/v) was used as mobile phase. This choice was due to the fact that for acidic compounds the selection of low pH buffer is preferred because the analyte dissociation is reduced and the interaction with the stationary phase is increased. In addition, ACN was selected as starting organic modifier because this solvent allows a relatively high EOF, even at low pH, which is useful to obtain short analysis times [35,36]. Even if in such experimental conditions KT and HK co-eluted, the analysis time was actually very short (about 5 min) and the other peaks were well resolved. Thus, this mobile phase was considered as a suitable starting point to improve method performance.

3.1. Effect of pH

Retention of KT impurities, being neutral molecules, was determined purely by chromatographic parameters. On the other hand, retention of KT, being a charged analyte, was additionally influenced by electrophoresis. Consequently, changes in mobile phase pH were fundamental to reaching the optimal conditions because this parameter might modify both EOF and KT dissociation, and the effect of pH could effectively be used in tuning KT selectivity.

The pH range considered in this study was 2.5–4.5. In fact, the use of pH higher than 4.5 led to a high delay of KT retention time and to a considerable broadening of KT peak shape, thus this value was selected as the upper limit of pH range. Fifty millimolar ammonium formate buffer was employed to evaluate the range 2.5–3.5, while the range 4.0–4.5 was covered by means of a 50 mM ammonium acetate buffer. In both cases, a mixture of 50 mM buffer of the proper pH–water–ACN (10:10:80, v/v/v) was employed as mobile phase.

As shown in Fig. 1, the neutral impurities' behaviour was probably influenced only by the absorption to the stationary phase. The relative migration order (HK as first eluting peak, KK and finally DK) remained the same throughout

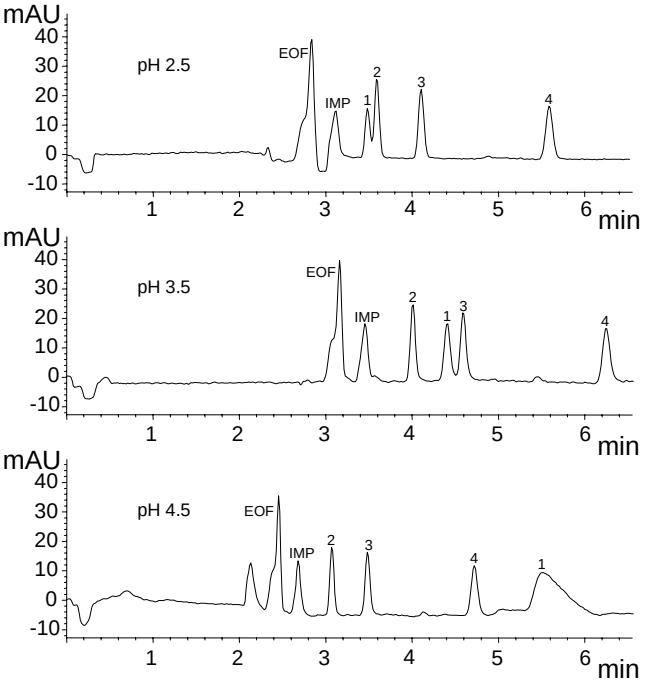


Fig. 1. Separation of (1) KT, (2) HK, (3) KK, (4) DK using different pH values. EOF, thiourea peak; IMP, unknown impurity. Capillary, 33.0 cm (effective length 24.5 cm) × 100 μm i.d. packed with LiChrospher 100 RP-18 (5 μm); mobile phase, mixture of 50 mM ammonium formate buffer (pH 2.5 and 3.5) or 50 mM ammonium acetate buffer pH 4.5–water–acetonitrile (10:10:80, v/v/v); voltage, 25 kV; temperature, 20 °C; pressure (both sides), 8 bar; injection, 12 bar for 12 s of 0.04 mg/mL of each compound + a plug of mobile phase at 12 bar for 12 s. Detection wavelength, 195 nm.

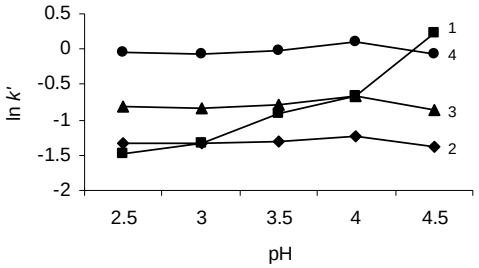


Fig. 2. Logarithmic retention factor ($\ln k'$) of (1) KT, (2) HK, (3) KK, (4) DK vs. mobile phase pH. For other experimental conditions, see Fig. 1.

the study and the three related substances were baseline separated from each other at all pH, values examined. On the other hand, a general increase of KT retention time was observed increasing pH, together with an increasing peak tailing effect from about pH 4.0 to upper pH values. KT behaviour was probably due to both the negative charge of the analyte, which has a self-mobility opposite to that of EOF (KT dissociation percentage increases increasing pH, from about 9% at pH 2.5 to about 91% at pH 4.5), and the interaction with the stationary phase. As a result, at pH 2.5 KT was the first eluting peak, at pH 3.0 it co-eluted with HK, at pH 4.0 it co-eluted with KK, and at pH 4.5 KT was the last eluting peak. At this pH, resolution among all the analytes was baseline but KT peak shape was not satisfactory, showing a very low efficiency (5144 N m^{-1}, about 15 times lower than that measured at pH 3.5) which was not suitable for accurate quantitation.

In Fig. 2 the plot of the logarithmic function of the retention factor k' versus pH is reported. This plot shows that, as expected, KT $\ln k'$ increased increasing the pH, while $\ln k'$ of the impurities was not significantly affected throughout the pH range considered.

On the basis of the obtained results, pH 3.5, corresponding to baseline resolution values among all the analytes (R_{s1} (HK/KT), 3.47; R_{s2} (KT/KK), 1.53; R_{s3} (KK/DK), 11.76, respectively) and to a quite low analysis time (about 6.5 min) was selected as starting point to further carry on the optimisation. The effect of pH 3.5 sodium formate versus ammonium formate as mobile phase buffer was tested, and the two electropherograms obtained were very similar. The only significant difference detected was the resolution value between KT and KK, which was 1.53 using ammonium formate and decreased to 1.06 using sodium formate, leading to discard this buffer in favour of the first.

3.2. Effect of ACN concentration

The content of organic modifier in the mobile phase was changed, preparing different mixtures of 50 mM ammonium formate buffer pH 3.5, water and acetonitrile, keeping the final buffer concentration (5 mM) constant and adding different percentages of the organic modifier from 50 to 80% (v/v). Excessively long analysis times were observed at ACN

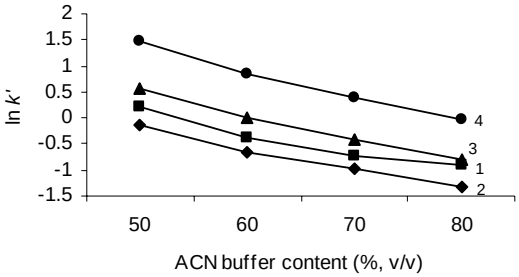


Fig. 3. Effect of % of acetonitrile present in the mobile phase on ln k' of (1) KT, (2) HK, (3) KK, (4) DK. Mobile phase, ammonium formate pH 3.5/ACN at different concentrations (the final concentration of ammonium formate was 5 mM). For other experimental conditions, see Fig. 1.

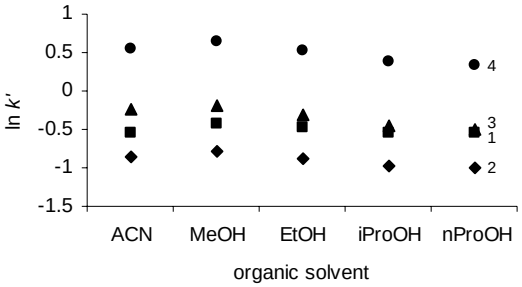


Fig. 4. Effect of organic modifier type on ln k' of (1) KT, (2) HK, (3) KK, (4) DK. Mobile phase, mixture of 50 mM ammonium formate buffer pH 3.5–water–different organic solvents–acetonitrile (10:20:10:60, v/v/v). For other experimental conditions, see Fig. 1.

concentrations lower than 50%. The change of acetonitrile concentration did not influence the migration order of the analytes.

The increase of ACN concentration in the mobile phase caused an increase of EOF (from 1.24×10^{-4} to 1.70×10^{-4} cm^2 V^{-1} s^{-1} at 50 and 80% ACN, respectively) and a lower analyte retention on the stationary phase, which resulted in a marked decrease of the retention times. This is also proved by the data reported in Fig. 3, where ln k' of each analyte is plotted against ACN concentration. ln k' of all studied compounds decreased by increasing the organic modifier concentration with an almost linear relationship, quite similar to that achieved in HPLC reversed-phase mode, due to the different partitioning between the stationary and mobile phase.

Selectivity (α) was generally found not significantly influenced by the organic modifier content (results not shown).

The increase of ACN concentration caused a general decrease of the resolution of all studied compounds, with the only exception of R_{s_1} (HK/KT). R_{s_2} (KT/KK) and R_{s_3} (KK/DK) values decreased almost linearly from 8.58 to 1.35 and from 24.27 to 11.88, respectively. R_{s_1} values decreased from 6.39 (50% ACN) to 2.94 (70% ACN), while the increase from 70 to 80% ACN caused an increase of resolution up to 3.56, mainly due to the higher efficiency of KT peak.

On the whole, the highest observed values of efficiency (N m^{-1}) for all the peaks apart from DK were obtained with 50% ACN (HK, $N = 97\,227$; KT, $N = 78\,068$; KK, $N = 121\,503$; DK, $N = 87\,808$). However, considering the high analysis time observed at this concentration (about 23 min), we decided to set the organic modifier concentration at 70%. Under these conditions, efficiency was still satisfactory. In fact, this latter decreased by about 0.1% for HK (97 177); 10.0% for KT (70 280); 8.8% for KK (110 839), and increased by about 23.9% for DK (108 827) with respect to the values observed at 50% ACN.

3.3. Effect of the organic modifier type

The physical properties of the mobile phase constituted the basis for further studies in order to verify the effect of the organic modifier type present. The mobile phase was modified keeping constant ACN content (60%) and 10% of the following modifiers were added to the mobile phase mixture: methanol, ethanol, isopropanol and *n*-propanol.

Adding these different organic modifiers some, but limited decrease of EOF was observed with respect to the use of acetonitrile only. Higher retention times (leading to analysis times ranging from about 10.5 to 12.0 min) were noticed and thus the choice of ACN as the only organic modifier was confirmed. The values of current observed did not change markedly; in fact, the lowest and the highest recorded values were 4.0 and 4.3 μA, obtained adding isopropanol and methanol, respectively.

A general decrease of ln k' was noticed going through MeOH, EtOH, iProOH and nProOH, respectively, due to the higher affinity of the compounds with the mobile phase (Fig. 4). Considering the organic modifiers in the same order, the difference between ln k' of KT and KK decreased, and this behaviour was confirmed also by the decrease of α_2 and R_{s_2} between KT and KK peaks (results not shown). In fact, adding MeOH and EtOH these two peaks were still baseline resolved, while adding iProOH and nProOH they were partially overlapped, with resolutions of 1.25 and 0.86, respectively.

3.4. Investigation of injection parameters and voltage

When adapting the separation method to the application to real samples, in order to determine all impurities and to obtain a sufficient sensitivity, a large loading of the main compound was required and the best CEC conditions were determined to be 12 bar × 60 s for sample injection and 0.1 mg/mL for KT test concentration.

When all the compounds were set at 0.1 mg/mL, a baseline resolution was observed for all the peaks. However, the KT peak showed a peak fronting effect, probably due to overloading. Concentration of KT could not be diluted without provoking a fall in sensitivity for the impurities, thus we tried to improve the efficiency of the peak making two attempts. The first was using a buffer with a negative counter-ion possessing a mobility comparable to KT, and ammonium citrate

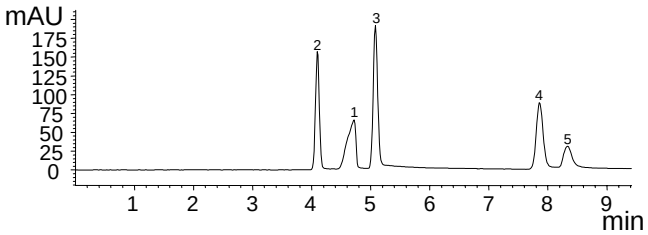


Fig. 5. Electrochromatogram of (1) KT, (2) HK, (3) KK, (4) DK, (5) FL under the optimized conditions. Mobile phase, mixture of 50 mM ammonium formate buffer pH 3.5–water–acetonitrile (10:20:70, v/v/v). Voltage, 30 kV; temperature, 20 °C; pressure (both sides), 8 bar; injection, 12 bar for 60 s of 0.1 mg/mL of each compound + a plug of mobile phase at 12 bar for 12 s. Detection wavelength, 323 nm.

was selected for this aim. However, this approach was not effective because this buffer in these conditions seemed to be unsuitable for CEC analyses for technical reasons related to current instability and bubble formation.

The second attempt consisted in increasing voltage from 25 to 30 kV and in this way KT peak shape was improved and found satisfactory for the quantitative analysis. The current raised to about 5.8 μA but stayed acceptably low, thus we decided to set voltage at 30 kV. In Fig. 5 the typical electrochromatogram obtained applying the optimal conditions is reported when all the compounds are at a concentration of 0.1 mg/mL and the detection wavelength is set at 323 nm.

3.5. Method validation

Validation of the method was performed using 0.1 mg/mL as KT test concentration and 323 nm as detection wavelength. This wavelength is near (or actually corresponds to) the maximum wavelength for all the analytes and gives the possibility of reducing the baseline noise and obtaining higher values of S/N ratio, with the consequence of lowering limits of detection (LOD) and quantification (LOQ) values.

The internal standard method was used to correct errors which are introduced by variable injection volume, voltage or EOF, and FL (0.1 mg/mL) was employed as internal standard.

3.5.1. Selectivity

In order to assess the selectivity of the method, a standard mixture containing 0.1 mg/mL of each of the analytes was analysed and separately spiked with each of the compounds. The identity of the peaks was additionally confirmed by analysing standards of each analyte individually and detecting their spectra with the DAD. All the peaks were baseline resolved and the resolution values measured were the following ($n = 4$, $\alpha/2 = 0.025$): R_{s_1} (HK/KT), 3.05 ± 0.52; R_{s_2} (KT/KK), 2.20 ± 0.41; R_{s_3} (KK/DK), 15.92 ± 0.23; R_{s_4} (DK/FL), 1.80 ± 0.25. The tablet excipients were analysed applying the optimised conditions and no interference was detected, thus this method was deemed suitable for application to the drug product.

Table 2
Within-day and between-day precision data for retention time and peak area ratios for a sample of 0.1 mg/mL KT, HK, KK, DK and FL (internal standard)

	R.S.D. (%, $n = 6$)			R.S.D. (%, $n = 18$)
	Day 1	Day 2	Day 3	Between days 1, 2 and 3
Peak area ratios				
A_{HK}/A_{FL}	2.60	2.99	2.95	3.96
A_{KT}/A_{FL}	1.77	2.31	1.62	5.36
A_{KK}/A_{FL}	1.48	1.94	2.14	4.11
A_{DK}/A_{FL}	1.48	1.55	2.75	3.35
FL retention time	1.41	0.67	3.14	3.07

A_{KT}, KT area; A_{HK}, HK area; A_{KK}, KK area; A_{DK}, DK area; A_{FL}, FL area.

3.5.2. Retention time and peak area repeatability

The standard mixture 0.1 mg/mL for each of the compounds was analysed six times in order to verify the repeatability (within-day precision) of the method in terms of retention times and peak area ratios. The same mixture was analysed over 3 days to evaluate intermediate precision (between-day precision). The obtained results are reported in Table 2.

An unusually high R.S.D. value was noticed for FL retention time (day 3), but it is believed to be mainly dependent on changes in room temperature (in fact, the carousel was not thermostated).

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

For each of the three impurities, LOD and LOQ were determined from three and ten times the signal-to-noise ratio (S/N) values, where the noise was calculated by Agilent Technologies ChemStation software.

The values of LOD were: HK, 0.0004 mg/mL (0.4%, w/w); KK, 0.0003 mg/mL (0.3%, w/w); DK, 0.0004 mg/mL (0.4%, w/w). The values of LOQ were: HK, 0.0012 mg/mL (1.2%, w/w); KK, 0.0010 mg/mL (1.0%, w/w); DK, 0.0013 mg/mL (1.3%, w/w).

Fig. 6 shows the electrochromatograms corresponding to LOD and LOQ measurements.

3.5.4. Linearity

The KT calibration graph was linear in the concentration range 0.07–0.13 mg/mL (70–130% of the nominal test concentration). Linearity for the impurities was evaluated in the following ranges, where the lower limits corresponded to the respective LOQ values: HK, 0.0012–0.0120 mg/mL (1.2–12.0%, w/w); KK, 0.0010–0.0100 mg/mL (1.0–10.0%, w/w); DK, 0.0013–0.0127 mg/mL (1.3–12.7%, w/w).

Linearity data are shown in Table 3.

3.5.5. Accuracy and precision

Accuracy and precision were assessed for each analyte at three concentration levels which covered the linearity range,

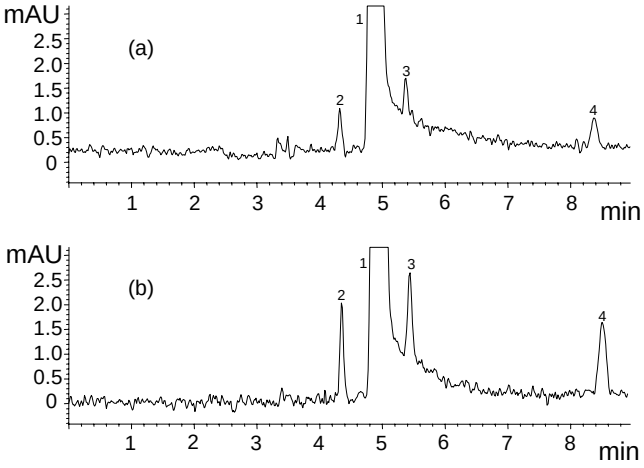


Fig. 6. Electrochromatograms obtained by injecting a working standard mixture containing 0.1 mg/mL of (1) KT with (2) HK, (3) KK, (4) DK concentrations set at the respective (a) LOD values and (b) LOQ values. For other experimental conditions, see Fig. 5.

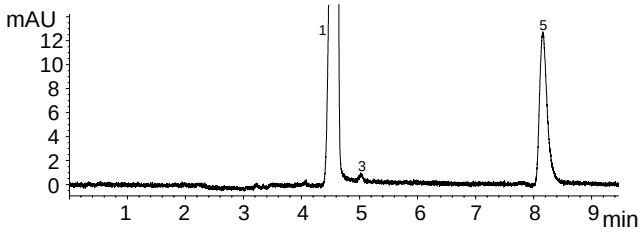


Fig. 7. Analysis of a real sample of Lixidol-coated tablets under the optimal conditions (see Fig. 5). (1) KT, (3) KK, (5) FL.

performing three replicates at each level. The obtained results are shown in Table 4.

3.6. Applications

The optimised method was applied to the assay of Lixidol-coated tablets and Fig. 7 shows the electrochromatogram relative to the analysis of the real sample.

The analysis was performed in quadruplicate and the recovery of KT was in agreement with the 10 mg labelled content ($\alpha/2 = 0.025$, recovery $98.5 \pm 0.8\%$, R.S.D. 0.5%). Among the related substances, it was possible to notice the presence of KK only, at a concentration under the LOD.

Table 4
Accuracy and precision data for the assay of KT and KT related substances ($n = 3$, $\alpha/2 = 0.025$)

Analyte	Concentration (mg/mL)	Accuracy (recovery, %)	Repeatability (R.S.D., %)
KT	0.0760	99.0 ± 3.5	1.4
	0.1000	102.8 ± 2.5	1.0
	0.1240	98.2 ± 4.5	1.8
HK	0.0020	103.1 ± 5.8	2.2
	0.0066	98.3 ± 1.9	0.8
	0.0112	97.3 ± 7.9	3.3
KK	0.0018	98.9 ± 3.5	1.4
	0.0055	104.4 ± 10.1	3.9
	0.0092	103.0 ± 4.7	1.8
DK	0.0021	97.2 ± 7.2	3.0
	0.0070	97.3 ± 3.3	1.4
	0.0119	96.3 ± 8.8	3.7

3.7. Comparison between MEKC method and CEC method

The drugs quality control is mainly performed using HPLC and CE techniques. This paper has shown how CEC has the potentialities to be effectively used in the purity assay of pharmaceuticals.

Both the MEKC [34] and CEC methods developed in our lab for the KT purity assay can in principle be routinely utilized in pharmaceutical quality control. The first requires about 6 min for the analysis, and the second less than 9 min. Preconditioning of the capillaries is more time consuming for CEC: this waste of time could be a limiting factor especially in phase of method optimisation, when the change of experimental conditions is often required, but could be easily amortized in case of routine analysis. On the other hand, the CEC method presents a higher selectivity, as it makes it possible to easily change the retention order of the compounds by changing pH of the mobile phase.

As regards the validation parameters of the two developed methods, the performances are comparable in terms of accuracy and precision (R.S.D. and bias values for all the analytes are below 5%). As regards sensitivity, LOD and LOQ for the impurities are comparable in terms of absolute values (i.e., concentrations of the analytes) but ten times lower in terms of percentage (w/w) for the MEKC method (about 0.1% or less).

It is worthwhile to note, however, that the choice of a volatile CEC mobile phase as formate buffer could afford

Table 3
Linearity data obtained for KT and KT related substances ($n = 7$, $k = 2$)

Analyte	Range (mg/mL)	a	s_a	b	s_b	S.E.	R^2	R^2_{cv}
KT	0.0700–0.1300	2.4495	0.0155	−0.0349	0.0158	0.0031	0.9995	0.9994
HK	0.0012–0.0120	1.5637	0.0321	0.0156	0.0024	0.0012	0.9950	0.9930
KK	0.0010–0.0100	1.2783	0.0265	0.0114	0.0017	0.0008	0.9949	0.9932
DK	0.0013–0.0127	0.6034	0.0099	0.0101	0.0008	0.0004	0.9968	0.9958

Regression equation, $y = ax + b$; s_a, standard deviation for the slope; s_b, standard deviation for the intercept; S.E., standard error.

the use of a MS detector with a consequent high increase of both sensitivity and selectivity.

4. Conclusions

The data presented suggest the alternative use of CEC as a purity assay for pharmaceuticals. This technique is very versatile, making separation possible of both neutral and charged species, and offers the advantages of using small sample and mobile phase volumes, as well as small amounts of stationary phases. Moreover, fast analysis times and high separation efficiencies are achieved.

The packed C_{18} capillary used in this study, produced in our laboratory, gave a stable performance for a relatively long time, as for about 300 runs the observed efficiency and repeatability showed no remarkable changes.

The optimisation step made it possible to select a mixture of 50 mM ammonium formate pH 3.5–water–acetonitrile (10:20:70, v/v/v) as mobile phase, providing a relatively high EOF. Applying the optimised conditions, all peaks were baseline separated and the analysis was performed in less than 9 min. The method was validated and effectively applied to the quantitative analysis of KT and its impurities in coated tablets.

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