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Development of a capillary electrophoresis method for the assay of ramipril and its impurities: An issue of cis–trans isomerization

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ABSTRACT

The development of a rapid and selective capillary electrophoresis method for the quantitation of ramipril and its eight main impurities in pharmaceutical dosage form is described. Ramipril and three of its impurities contain a proline-similar moiety which causes in solution the presence of interconverting cis–trans isomers with respect to the amide bond. The interplay between electrophoretic migration and isomerization may yield the presence of an undesired interconversion zone between the two isomer peaks in the electropherogram, depending on the experimental conditions. Different capillary electrophoresis operative modes and pseudostationary phases were evaluated, both in normal and reverse polarity, in order to find the essential analytical parameters which could make it possible to overcome this issue and thus accurately quantify the analytes. The best results were obtained by using microemulsion electrokinetic chromatography in reverse polarity, where all the compounds which undergo cis–trans interconversion migrate as a single narrow peak. Experimental design led to identification of the following optimised conditions: background electrolyte, microemulsion made by 88.95% of 90 mM phosphate pH 2.5, 1.05% of *n*-heptane and 10.00% of SDS/*n*-butanol in 1:2 ratio; voltage, −26 kV; temperature, 17 °C. Applying these conditions, the baseline separation of the analytes was obtained in about 10 min. Validation of the method following ICH guidelines was carried out and the procedure was applied to a real sample of ramipril tablets.

1. Introduction

Ramipril (RM, CAS 87333-19-5) is an angiotensin-converting enzyme (ACE) inhibitor with a zwitterionic nature, used in the treatment of hypertension, heart failure and after myocardial infarction to improve survival in patients with clinical evidence of heart failure [1]. According to the information given by the drug manufacturer, Sanofi-Aventis (Sisteron, France), in dosage form RM tablets eight main impurities may be found, which in turn present neutral, acidic or zwitterionic properties. The chemical structures of RM and its impurities, coded as I_A, I_B, I_C, I_D, I_F, I_K, I_L, I_M following the Ph. Eur. monograph [2], are reported in Fig. 1.

For the determination of RM alone or in combined dosage forms several analytical procedures have been described, including spectrophotometry [3–7], spectrofluorimetry [7], atomic absorption [4,6], conductometry [8], voltammetry [9,10], flow injection analysis [11], HPTLC [12,13] and HPLC [3,7,12,14–16]. In biological fluids, determination of RM alone or with its metabolite ramiprilat has been performed by voltammetry [9,10], spectrofluorimetry [5], GC–MS [17] and LC–MS/MS [18–21].

For the simultaneous analysis of RM and its related substances HPLC methods have been developed, taking into consideration samples constituted by different mixtures of analytes, represented by two RM precursors [22] and more recently by ten RM related substances, including I_A, I_B, I_C, I_D and I_F [23]. The official HPLC methods described in the current Ph. Eur. [2] and USP [24] are capable of determining only four specified related substances, namely I_A, I_B, I_C and I_D.

Capillary electrophoresis (CE) has been extensively used for impurity analysis of pharmaceuticals [25]. To our knowledge, analysis of RM by CE methods has only concerned the separation of mixtures of different ACE inhibitors [26–29], without focusing on the simultaneous determination of RM and its impurities. Therefore, the novelty of this study consists in the development of a CE method able to fulfil this purpose, which could be routinely used for quality control of the pharmaceutical dosage form. As drug registration authorities are interested in following up on pharmaceutical impurities in the range as low as 0.01–0.10% w/w with respect to the main component [25], the requirements of the method were both the baseline resolution of the compounds and the possibility to quantify the impurities at the 0.1% w/w level or lower.

Due to the different acid–base properties of analytes, the selection of a suitable pseudostationary phase was approached, in order to take advantage of a double separation mechanism based on both

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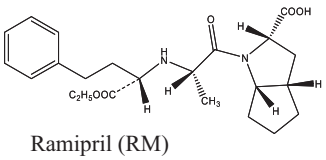
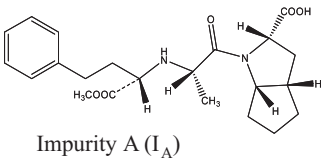

Ramipril (RM)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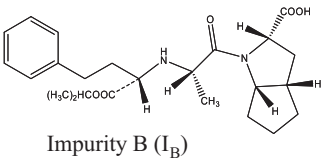
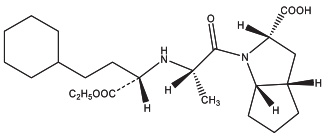
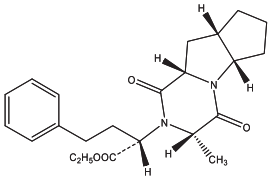
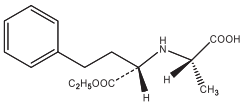
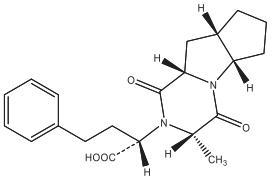
Ramipril methyl ester

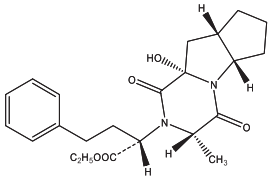

Impurity B (I_B)
Ramipril isopropyl ester


Impurity C (I_C)
Hexahydroramipril


Impurity D (I_D)
Ramipril diketopiperazine


Impurity F (I_F)
(*S*)-2-[[(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-propanoic acid]


Impurity K (I_K)
Ramipril diketopiperazine acid


Impurity L (I_L)
Ramipril hydroxydiketopiperazine

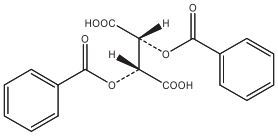

Impurity M (I_M)
(2*R*,3*R*)-2,3-bis(benzoyloxy)succinic acid

Fig. 1. Chemical structures of the analytes.

the different electrophoretic mobilities of solutes and their different partitioning in the retentive phase [30]. Moreover, RM and I_A, I_B, I_C contain a proline-similar moiety, which causes the presence in solution of two cis–trans isomers that interconvert around the amide bond [31]. The issue of cis–trans isomerization of small peptides and ACE inhibitors has been recently addressed for capillary electrophoresis, concerning both the separation of the isomers and/or kinetic studies [31–39]. In general, the rates of interconversion of this kind of isomer are on the order of seconds to minutes [31], leading to the possible interference between electrophoretic migration and reaction of isomerization, ruled by the Damköhler number (*Da*). This is defined as the ratio of the residence time of the migrant in the capillary (mean migration time of the two conformers) to the relaxation time of the reaction. At low *Da* values the cis–trans isomers are separated with baseline resolution, while at high *Da* values they elute as a single peak. Intermediate values of *Da* lead to a typical electrophoretic pattern where two individual peaks of the separated conformers are connected by a plateau consisting of a mixture of both conformers, thus making an accurate determination of the compounds not possible [34].

Due to the complexity of the analytical problem, the choice of a proper CE operative mode represented a significant challenge. For this reason, a large part of the preliminary experiments, run with the aid of the experimental design [40], were dedicated to the selection among different pseudostationary phases based on micelles, mixed micelles or microemulsions, with or without additives, operating both in normal and reverse polarity.

In this work the best results, among the evaluated operative modes, were obtained by applying microemulsion electrokinetic chromatography (MEEKC) in reverse polarity; in these conditions cis and trans isomers migrate as a single narrow peak. The MEEKC method was optimised by applying a multivariate strategy, first employing mixture design [41] in order to identify the optimal composition of the microemulsion and then response surface methodology [40] to identify the optimal values of the independent factors. Validation of the method was then performed following ICH Guidelines [42].

2. Materials and methods

2.1. Chemicals and reagents

Sodium tetraborate decahydrate (borax) was from BDH Laboratory Supplies (Poole, UK). Boric acid, 86.1% phosphoric acid, sodium hydrogen carbonate, methanol (HPLC grade), sodium dodecyl sulphate (SDS), *n*-heptane, *n*-butanol, sodium cholate, sodium deoxycholate, sodium taurocholate, sodium taurodeoxycholate, 3-(N,N-dimethylmyristylammonio)propanesulfonate (MAPS), polyoxyethylene (23) lauryl ether (Brij 35), polyoxyethylene (20)-sorbitan monolaurate (Tween 20), (2-hydroxypropyl)-α-cyclodextrin (HPαCD), (2-hydroxypropyl)-β-cyclodextrin (HPβCD), (2-hydroxypropyl)-γ-cyclodextrin (HPγCD), methyl-β-cyclodextrin (MβCD), heptakis(2,6-di-O-methyl)-β-cyclodextrin (DMβCD), heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin (TMβCD) and all the other chemicals used were purchased from Sigma–Aldrich (St. Louis, MO, USA).

The reference standards of RM and RM impurities were kindly supplied by Sanofi-Aventis (Sisteron, France), as well as the excipients hydroxypropyl methylcellulose, pregelatinized maize starch, mycrocrystalline cellulose and sodium stearyl fumarate. Triatec® tablets (Sanofi-Aventis), labelled to contain 10 mg RM, were purchased in local pharmacies. The reference standard of flufenamic acid (FL), employed as internal standard, was purchased from Sigma–Aldrich.

Ultrapure water used for the preparation of solutions and running buffers was provided by a Simplicity 185 system (Millipore, Billerica, MA, USA) after an electrodeionisation treatment using an Elix system (Millipore).

2.2. Solutions, microemulsions and sample preparation

Standard stock solutions of RM (10 mg mL^{-1}) and of FL (internal standard, 2 mg mL^{-1}) were prepared in 0.1 M $NaHCO_3$; standard stock solutions of RM impurities (2 mg mL^{-1}) were prepared in methanol. All the prepared stock solutions were stored at 4 °C

for one week. Working standard solutions were prepared daily by appropriate dilution with water in a vial to 500 μL in order to obtain the desired final concentration values of the different compounds.

Buffers were prepared by mixing an adequate volume of the proper 0.5 M acid, adjusting pH with 1 M sodium hydroxide and filling up to volume with water. In the case of borax buffer, the desired pH was obtained by adding 1 M NaOH or 1 M HCl.

Pseudostationary phases made by micelles and mixed micelles were prepared by weighing the proper amount of the surfactant or the two surfactants, to which the appropriate volume of buffer was added. Pseudostationary phases constituted by microemulsion oil droplets were obtained by sequentially mixing in a beaker proper amounts of aqueous phase, cosurfactant (*n*-butanol), surfactant (SDS) and finally oil (*n*-heptane), taking care to add each component only after reaching a complete dissolution of the previously mixed compounds. All microemulsions were prepared on a w/w basis. For normal polarity CE analysis, the aqueous phase of the microemulsion was composed of 10 mM borax pH 9.2, while for reverse polarity CE analysis the aqueous phase was constituted by phosphate buffer pH 2.5 in the concentration range 70–100 mM. The considered percentage of the microemulsion components during optimisation phase was 88.00–93.90% for the aqueous phase, 0.10–2.00% for the oil phase and 6.00–10.00% for the mixture surfactant/cosurfactant in 1:2 ratio.

For tablet assay, 30 tablets were weighed and finely powdered. An accurately weighed portion of the powder, corresponding to about 200 mg RM, was transferred into a 25 mL beaker to which 10 mL methanol was added. The obtained mixture was stirred for 5 min, sonicated for 10 min and stirred again for 5 min. An aliquot of 1 mL of the mixture was then centrifuged and 100 μL of the supernatant were introduced into a 500 μL vial for analysis, together with 25 μL of FL standard stock solution and 375 μL of water, in order to obtain a RM test concentration of about 4 mg mL^{-1} and a FL concentration equal to 0.1 mg mL^{-1}.

2.3. Instrumentation and CE analysis

A Metrohm 691 pH Meter (Metrohm, Herisau, Switzerland) was used to measure pH. A multiple magnetic stirrer Multipoint HP15 (Variomag, Daytona Beach, USA) was used to stir microemulsions. Tablet assay mixtures were sonicated by a 300 Ultrasonik ultrasonic bath (Ney Company, Bloomfield, USA).

CE experiments were run on an Agilent Technologies ^{3D}CE system (Agilent Technologies, Waldbronn, Germany) which was driven by ^{3D}CE ChemStation software (Rev. A.09.01, Agilent Technologies) and had a UV–visible diode array detector and an air thermostating system. The analyses were carried out in uncoated fused-silica capillaries (50 μm inner diameter and 375 μm outer diameter, Composite Metal Services, Ilkley, UK) of different total lengths (64.5, 48.5, 33.0 cm). Among these, the 64.5 cm capillary was selected as optimal. The capillaries were cut using a Capillary Cleaving™ tool (Supelco, Bellefonte, PA, USA). The detection window was built-in by burning off the polyimide coating on the capillary using The Windowmaker™ (MicroSolv, Postnova Analytics, Landsberg/Lech, Germany).

The value and polarity of voltage and the value of temperature were set as described in the text. Detection wavelength was set at 210 nm; injection of the sample was hydrodynamically performed for 10 s at 50 mbar, followed by a background electrolyte (BGE) plug for 10 s at 50 mbar. In the optimised conditions, voltage was set at −26 kV (rise time 0.20 min, reverse polarity) and temperature was set at 17 °C. The optimised BGE consisted of a microemulsion made of 88.95% 90 mM phosphate buffer pH 2.5, 1.05% *n*-heptane and 10.00% SDS/*n*-butanol in 1:2 ratio.

Every new capillary was initially conditioned with 1 M NaOH and with water for 5 min each. Between the electrophoretic runs, the capillary was rinsed with methanol (2 min), 1 M NaOH (1 min), 0.1 M NaOH (1 min), water (1 min) and BGE (4 min).

2.4. Calibration curves, calculations and software

Calibration curves were obtained using FL as internal standard at a constant concentration value of 0.1 mg mL^{-1} and by plotting the ratio of analyte/internal standard corrected peak areas *versus* analyte concentration. Five samples at different concentration values were analyzed, carrying out two electrophoretic runs for each of the five samples. The RM regression curves were evaluated in the range 40–120% with respect to the test concentration, corresponding to 1.6–4.8 mg mL^{-1}. The considered ranges for the impurities were from the respective LOQ to the 1% w/w with respect to the main component: I_A, 0.0020–0.0400 mg mL^{-1}; I_B, 0.0020–0.0400 mg mL^{-1}; I_C, 0.0040–0.0400 mg mL^{-1}; I_D, 0.0012–0.0400 mg mL^{-1}; I_F, 0.0030–0.0400 mg mL^{-1}; I_K, 0.0020–0.0400 mg mL^{-1}; I_L, 0.0040–0.0400 mg mL^{-1}; I_M, 0.0030–0.0400 mg mL^{-1}.

R_h, measurement of the interference between isomerization and electrophoretic migration, was calculated as the ratio between the height of the interpeak reaction zone and the height of the peak corresponding to the faster migrating conformer [32].

The experimental design software used to set-up designs and to perform analysis of variance (ANOVA) was NEMROD-W [43].

3. Results and discussion

In order to perform an accurate quantitation of the analytes, the cis–trans interconversion zone in the electropherogram had to be eliminated by selecting suitable experimental conditions leading to a low or a high value of Damköhler number. The value of *Da* depends on different experimental parameters, including chemical nature of the system (BGE, electro-osmotic flow (EOF), electrophoretic mobilities of the migrant molecules), effective length of the capillary, applied electric field and temperature [32]. Thus, the first series of experiments was intended to establish the analytical requirements of the method, namely operative mode and pseudostationary phase, able to avoid the peak splitting of the rotamers. Before running the study, it was clear that from a practical point of view of impurity profiling, two main potential issues could arise when working with a low *Da*, i.e. a reduced sensitivity for the impurities and an increased complexity of the separation, both of them due to cis–trans peak splitting. In any case, an in-depth investigation of the effect of various operating conditions on the electrophoretic pattern was deemed of valuable interest due to its novelty, and the possibility of reaching the quantitative aim by working with a low *Da* was kept for the study.

3.1. Normal polarity

The first series of electrophoretic runs was carried out operating in normal polarity, using negatively charged pseudostationary phases. Under these conditions the cathode is positioned at the outlet side of the capillary and a basic buffer is required in order to achieve a high EOF and thus draw to the detector all the analytes which possess negative or zero charge.

When using SDS-based micelles as pseudostationary phase (micellar electrokinetic chromatography, MEKC), RM, which has to be analyzed at a test concentration value at least one hundred times above the concentration value of the impurities, migrated as a pair of peaks corresponding to the rotamers. These two peaks were connected by a broadened migration zone generated by the mixture of the isomeric forms (Fig. 2a). In order to improve the separation of the conformers of RM simultaneously with those generated by

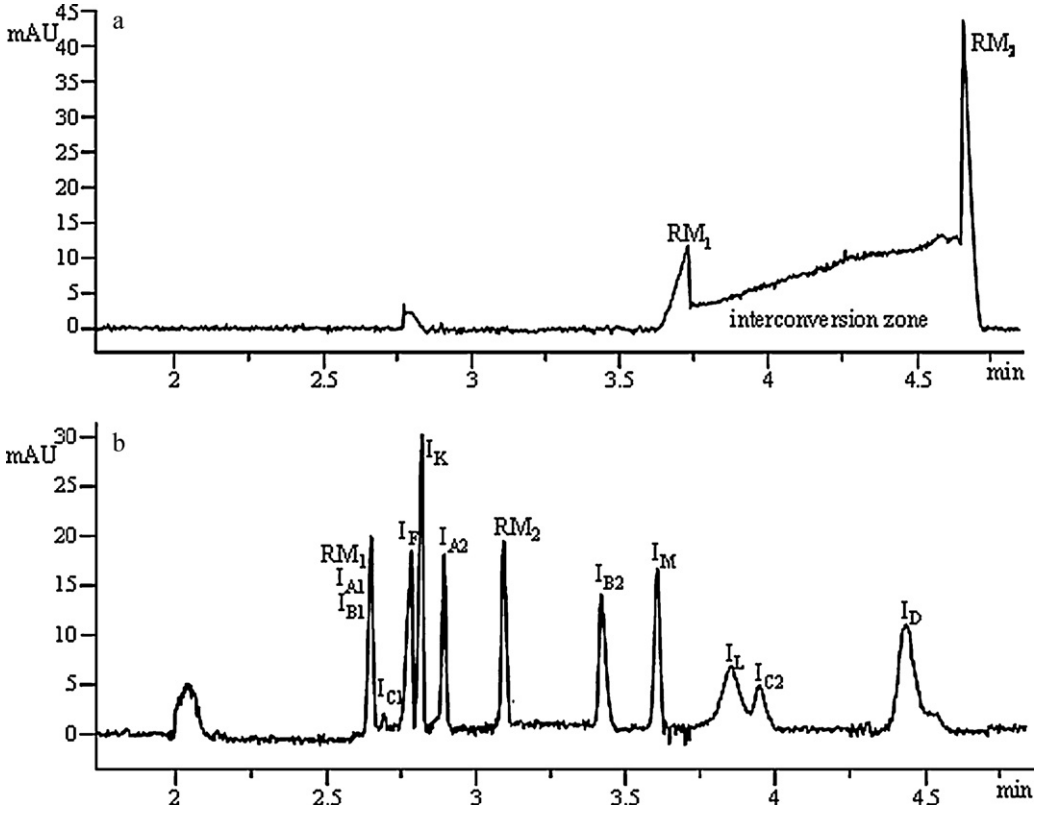


Fig. 2. Electropherogram of (a) 2 mg mL^{-1} RM and (b) 0.04 mg mL^{-1} analytes. BGE, 20 mM borate pH 9.50, 30 mM SDS; detection wavelength, 210 nm. (a) Capillary length, 64.5 cm; voltage, 30 kV; temperature, 30 °C. (b) Capillary length, 48.5 cm; voltage, 25 kV; temperature, 20 °C. Symbols as in Fig. 1.

the proline-containing impurities (namely I_A, I_B and I_C), separation systems using different running buffers with pH values in the 8.50–10.00 range were examined. The best results were obtained with 20 mM borate buffer at pH 9.50–9.75; however the selectivity of this separative system was not sufficient, mainly due to a critical zone at the beginning of the electropherogram, corresponding to the migration of the faster isomers of RM, I_A, I_B and I_C (Fig. 2b).

In order to rapidly verify the possibility of obtaining high *Da* values for the analytes, temperature was increased from 20 °C up to 35 °C. In fact, this parameter is the most important factor acting on the Damköhler number, as it exponentially affects the kinetics of the reaction and also influences the viscosity of the medium [32]. At 35 °C only I_B was led to migrate as a single sharp peak, while setting higher temperature values an extremely detrimental and general loss of efficiency was evidenced, which was not compatible with the possibility of obtaining a sufficient sensitivity for the impurities.

Therefore, attention was focused on obtaining or improving the separation of the peaks, as well as on eliminating the RM interconversion zone by lowering the related *Da* value. With this aim, variations of the borate/SDS composition were made; further, secondary equilibria based on host–guest complexation were introduced by addition of different neutral cyclodextrins (mentioned in Section 2.1, at concentrations values 15–30 mM), but no significant improvement in the electrophoretic pattern was obtained. The potential of mixed micellar electrokinetic chromatography (MMEKC) operative mode was also investigated, since by combining surfactants with different structural properties solute–micelle interactions can be effectively manipulated. Different combinations of surfactants were evaluated (bile salts, zwitterionic surfactants such as MAPS and non-ionic surfactants such as Brij 35 and Tween 20). Unfortunately, also in this case adequate separation of the peaks was not achieved.

Finally, a MEEKC experiment was run; in particular the microemulsion was made of 90.95% aqueous phase (10 mM borax pH 9.2), 1.05% oil phase (*n*-heptane), 8.00% surfactant/cosurfactant (SDS/*n*-butanol) in 1:2 ratio. This system had been previously recognised as having a wide versatility and a high resolving power [44–47], however in this case the results obtained were far from the desired performances.

3.1.1. Multivariate study of SDS-based MEKC

To verify the potential of SDS-based MEKC, a multivariate strategy was applied. The selected responses were the value of R_h, related to the Damköhler number, to be minimised, and the number of analytical peaks *n.p.*, related to the selectivity of the separation, to be maximised. Two different samples were considered: one containing 2 mg mL^{-1} RM, in order to measure R_h, and one containing all the analytes at 0.04 mg mL^{-1}, in order to measure *n.p.*

In a first screening phase the considered factors included borate concentration (15–25 mM), SDS concentration (20–40 mM), voltage (15–25 kV), total length of the capillary (33.0–48.5–64.5 cm), buffer pH (9.00–9.50–10.00) and temperature (16–20–24 °C). The responses were modelled according to a Free-Wilson model [40,48], and the model coefficients were estimated by a screening asymmetric matrix [40]. The responses were statistically treated [49] and graphic analysis of effects was used to detect the significant changes of factor values and to choose the suitable levels of the factors for the simultaneous optimization of the responses [50]. The following values for the factors were fixed: borate concentration, 20 mM; voltage, 25 kV; capillary length, 64.5 cm; temperature, 16 °C.

The effects of pH (9.50–10.00) and SDS concentration (30–70 mM) were more deeply investigated by means of response surfaces. The concentration of tetrahydrofuran (30–70 mM) was

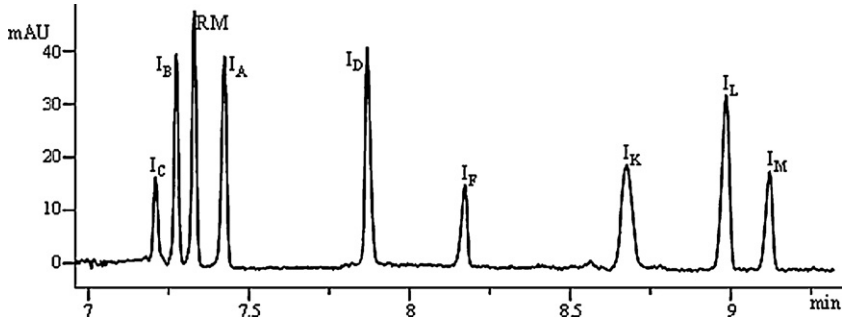


Fig. 3. Electropherogram of 0.04 mg mL^{-1} analytes. BGE, microemulsion composed by 90.95% of 100 mM phosphate pH 2.5, 1.05% of *n*-heptane, 8.00% of SDS/*n*-butanol in 1:2 ratio; capillary length, 64.5 cm; detection wavelength, 210 nm; voltage, −25 kV (reverse polarity); temperature, 20 °C. Symbols as in Fig. 1.

also introduced as a third factor, since its addition to the BGE caused an enlargement of the migration window and an increase of efficiency and symmetry of the RM second migrating peak. The coefficients of the quadratic model were estimated by a Box–Behnken design [51]. For both the responses, a net improvement with respect to the values measured in the screening phase was found. However, in spite of the obtained advances it was not possible to reach a satisfactory electropherogram. In fact, the model for *n.p.* resulted to be not significant, and this was probably due to practical problems encountered such as inversions and changes in the migration order of the peaks and peak collapsing [52]. As concerns R_h, the minimum value of the predicted response in the considered domain was still too high to eliminate the problems due to the interconversion zone.

3.2. Reverse polarity

When working in reverse polarity, the anode is positioned at the outlet side of the capillary. EOF should be strongly suppressed, as it would draw the analytes towards the cathode. This requirement can be fulfilled by using a high ionic strength buffer at low pH. The negatively charged pseudostationary phases draw to the detector all the analytes which possess positive or zero charge.

In this case, 100 mM phosphate buffer pH 2.5 was selected as the plain electrolyte to which micellar and microemulsion pseudostationary phases were added. As early as the first electrophoretic runs it was clear that the pH change of the BGE with respect to normal polarity had a crucial effect on cis–trans interconversion. In fact, when using an acidic pH all the compounds which undergo the cis–trans interconversion migrate as a single peak. A pH decrease was thus confirmed to be essential to avoid peak splitting [3], and this effect can be explained by a higher isomerization rate as previously reported [3,53].

When using SDS-based micelles as pseudostationary phase, the selectivity between RM, I_A, I_B, and I_C was very low, as the related peaks were almost completely collapsed. Thus this system was discarded.

On the other hand, the results obtained using a microemulsion as pseudostationary phase were very promising. The composition of the microemulsion was the same evaluated for MEEKC in normal polarity, with the only difference being the kind of aqueous phase, namely a 100 mM phosphate buffer pH 2.5 instead of 10 mM borax pH 9.2. The electropherogram obtained when analysing a mixture of all the analytes at a concentration value equal to 0.04 mg mL^{-1} showed a baseline resolution of all the compounds (Fig. 3). This separative system was thus selected as starting point for undertaking the optimization phase. The test concentration for RM in real samples was selected as 4 mg mL^{-1}, in order to assure LOQ values for the impurities at 0.1% or lower. The composition of the microemulsion and the effects of independent factors were then investigated using a multivariate approach in order to find the experimental conditions leading to a complete baseline separation of the analytes in a short analysis time.

3.2.1. Multivariate optimisation of MEEKC

Mixture design was employed to find the optimal composition of the microemulsion, allowing the complete separation of the analytes. In this kind of design, the considered response is a function of the relative proportions of the components of the mixture [41]. The selected responses were the critical resolution values between adjacent peaks.

The considered components were the aqueous phase (W), the oil phase (O) and the mixture surfactant/cosurfactant (S/CoS) in a constant ratio of 1:2. Their upper and lower constraints were the following: W, 88.00–93.90%; O, 0.10–2.00%; S/CoS 6.00–10.00%. A Scheffé special cubic regression model was hypothesized and the coefficients were estimated by a 13-run mixture design [40] already successfully employed in other studies [44,46]. Unfortunately, in this case the obtained electropherograms did not allow the considered responses to be statistically treated. In fact, in some experiments an overlap or inversion of the migration order of pairs of analytes occurred, making it not possible to univocally measure the resolution values between the considered peaks [52]. In any case, the best results were obtained using a microemulsion made of 88.95% W, 1.05% O, and 10.00% S/CoS, and this composition was selected as optimal.

In the subsequent step, the optimisation of the following independent factors was carried out by means of response surface methodology: concentration of phosphate buffer (BGE conc., 70–100 mM), voltage (*V*, 20–30 kV) and temperature (*T*, 16–20 °C). The selected responses were the critical resolution values R_1 (I_B/RM) and R_2 (RM/I_A), to be maximized, and the analysis time *t*, to be minimized. Doehlert design was employed in order to estimate the coefficients of the quadratic model relating the factors to the response and the corresponding experimental plan is reported in Supplementary Table S1. ANOVA [49] showed that the regression model was valid and significant for all the responses and this made direct investigation possible of the response surfaces, represented in Supplementary Fig. S1. The optimal values of the factors were identified by means of desirability function (*D*) [40]. Partial desirability functions were defined in order to obtain a baseline separation for R_1 and R_2 and a reasonable analysis time. From the graphical representation of total desirability function, shown in Supplementary Fig. S2, maximization of *D* was achieved by setting intermediate values of phosphate concentration and medium-high values of voltage. No particular constraint was evidenced for temperature, as all the values included in the domain, combined with suitable levels of phosphate concentration and voltage, could correspond to maximum desirability. Thus, temperature was set at 17 °C, a value able to increase the efficiency of the peaks related

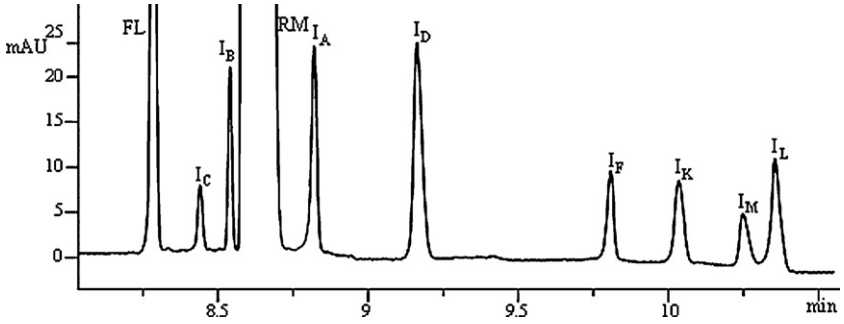


Fig. 4. Electropherogram of 4 mg mL^{-1} RM and 0.04 mg mL^{-1} RM impurities referring to optimal conditions. BGE, microemulsion composed by 88.95% of 90 mM phosphate pH 2.5, 1.05% *n*-heptane, 10.00% SDS/*n*-butanol in 1:2 ratio; capillary length, 64.5 cm; detection wavelength, 210 nm; voltage, −26 kV (reverse polarity); temperature, 17 °C. Symbols as in Fig. 1; FL, flufenamic acid.

to the impurities without any instrumental trouble in maintaining this temperature value. The optimal conditions corresponded to the following: phosphate concentration, 90 mM; voltage, −26 kV (reverse polarity); temperature, 17 °C. The typical electropherogram referring to the optimized conditions is reported in Fig. 4, showing the complete separation of the analytes in about 10 min, with a generated current of about −80 μA.

3.3. Validation and application

The optimized MEEKC method was validated following ICH Guidelines [42] using a test solution made of 4 mg mL^{-1} RM and 0.02 mg mL^{-1} RM impurities (0.5% w/w with respect to the main compound), unless described otherwise. Concentration of the internal standard was 0.1 mg mL^{-1} throughout the experiments.

Selectivity of the method was assessed by identifying the peaks by spiking and measuring the critical resolution values ($n = 4$, $\alpha/2 = 0.025$), corresponding to the following: R_1, 0.99 ± 0.12; R_2, 1.73 ± 0.16. The lack of interference by the excipients was checked by running a blank. In order to determine system repeatability, the test mixture was analyzed six times on three consecutive days, obtaining for corrected area ratios within-day RSD values ($n = 6$) ranging from 0.4 to 0.8% for RM and ranging from 1.3 to 3.4% for RM impurities. As concerns between-day RSD ($n = 18$), the obtained value for RM was 0.7%, while for RM impurities the values were included in the interval 1.5–3.4%. As regards analysis time, within-day RSD was from 0.5% to 3.6%, while between-day RSD was 4.2%.

For robustness testing the considered responses were the critical resolution values R_1, R_2 and analysis time t. The considered factors, together with their experimental range, centered on the optimized values, were the following: pH 2.4–2.6; phosphate concentration (BGE conc.), 88–92 mM; voltage (V), 25–27 kV; temperature (T), 16–18 °C. The factors were investigated by an eight-run Plackett–Burman design [40] and the experimental plan with the measured responses is reported in Table 1. Interpretation of effects was done graphically [50] and the only factors which could modify the method performances when varied in the considered domain were phosphate concentration and voltage. Both of them had a negative effect on analysis time and thus needed to be more strictly controlled when applying the procedure.

The LOD and LOQ of RM impurities were determined on the basis of S/N, considering a ratio of approximately 3:1 and 10:1, respectively. LOD/LOQ values were the following, respectively: I_A, 0.0010/0.0020 mg mL^{-1}; I_B, 0.0010/0.0020 mg mL^{-1}; I_C, 0.0023/0.0040 mg mL^{-1}; I_D, 0.0005/0.0012 mg mL^{-1}; I_F, 0.0012/0.0030 mg mL^{-1}; I_K, 0.0010/0.0020 mg mL$^{-1;}$ I_L, 0.0020/0.0040; I_M, 0.0018/0.0030 mg mL^{-1}. LOQ values were validated by performing eight replicates and by measuring RSD of corrected area ratios; the values obtained were included in the range 3.6–5.4%.

Table 1
Reverse polarity MEEKC robustness testing: Plackett–Burman experimental plan and responses.

Exp. no.	pH	BGE conc. (mM)	V (kV)	T (°C)	R_1[a]	R_2[b]	t (min)
1	2.6	92	27	16	0.92	1.73	9.51
2	2.4	92	27	18	1.03	1.71	9.21
3	2.4	88	27	18	0.86	1.90	10.26
4	2.6	88	25	18	0.78	1.66	10.76
5	2.4	92	25	16	0.92	1.68	10.64
6	2.6	88	27	16	1.07	1.79	10.32
7	2.6	92	25	18	0.82	1.84	10.56
8	2.4	88	25	16	0.93	1.72	11.62

[a] R_1, resolution between I_B and RM.
[b] R_2, resolution between RM and I_A.

The RM linearity range was 1.6–4.8 mg mL^{-1} (40–120% of the test concentration). The equation was $y = 5.3979x - 0.2689$ ($R^2 = 0.9989$), where y is the corrected area ratio RM/internal standard and x is the concentration of RM in mg mL^{-1}. The RM impurities linearity range was from the respective LOQ values to 1% w/w with respect to RM. The values of R^2 ranged from 0.9950 to 0.9982.

Accuracy and precision, as degree of repeatability, were established across the linearity range, using nine determinations over three concentration levels ($n = 3$, $\alpha/2 = 0.025$). RM recovery values ranged from $98.8 \pm 1.5\%$ to $101.3 \pm 3.1\%$, with a maximum RSD of 1.2%. RM impurities recovery values ranged from $96.4 \pm 7.4\%$ to $103.2 \pm 6.0\%$, with a maximum RSD of 4.9%.

System suitability limits were determined on the basis of the values of R_1 and R_2 measured during system repeatability study [54]. The accepted range for the performance criteria corresponded to the following: R_1, 0.87–1.06; R_2, 1.63–1.88.

The validated method was applied to run the analysis of a real sample composed of Triatec® tablets, labelled to contain 10 mg RM. Four analyses were performed ($n = 4$, $\alpha/2 = 0.025$), obtaining results in agreement with the declared content: assay, $98.7 \pm 1.5\%$; RSD, 1.0%. None of the impurities were detected, meaning that the concentration of impurities was lower than the respective limit of detection, thus confirming the expected safety of marketed tablets.

4. Conclusions

A rapid and selective CE method for the analysis of RM and its principal eight impurities in pharmaceutical dosage form was developed. Due to the different acid–base properties of the compounds and due to the interplay between migration and cis–trans isomerization of some of them, selection of the experimental conditions was extremely critical. The wide versatility of capillary electrophoresis made it possible to effectively solve the analytical problem. The performances of different operative modes and pseu-

dostationary phases were examined, both in normal and reverse polarity. Evaluation of the obtained electrophoretic patterns led to the selection of reverse polarity MEEKC under acidic conditions, where all the compounds, undergoing rapid isomerization, migrate as single peaks. Experimental design played a fundamental role both in the selection of the operative mode and in the reverse polarity MEEKC optimisation. Its use allowed an in-depth study of the effect of different factors on the electropherogram, obtaining a good quality of information with a limited number of experiments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.02.062.

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