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Research Article

Development of an enantioselective capillary electrophoretic method for the simultaneous determination of montelukast enantiomeric and diastereoisomeric forms and its main degradation product

A stereoselective CD-MEKC system has been developed for the quality control of Montelukast (MK), commercialized as a pure enantiomer. The proposed method is the first one that allows the simultaneous determination of MK, its enantiomeric form, diasteroisomers and its main degradation compound (MK sulphoxide). CD-MEKC system is composed of 10 mM SDS, 10 mM sulfobutylether-β-CD, 10 mM TM-β-CD, and 20 mM borate buffer at pH 9.0. Combination of these two CDs allows high baseline enantioresolution between MK and its enantiomeric impurity, but also, between the diasteroisomeric forms. Moreover, a multivariate design was applied to optimize operational parameters. The method was designed to meet with requirements of the official pharmacopoeias and fully validated according to international guidelines. Linearity of MK was demonstrated in the range from 10.0 to 100.0 μg/mL ($r^2 = 0.9908$) with a LOD and LOQ of 0.30 and 0.90 μg/mL, respectively. Intra and interday precision were evaluated and RSD values were below 2%, and also, accuracy expressed as percentage of recovery was in a range from 99.0 to 101.9 for the three assayed levels. The method allows determining 0.02% w/w of the enantiomeric and diasteroisomeric impurities, and 0.01% w/w of MK sulphoxide. Robustness was evaluated by a Plackett and Burman design. Finally, the CD-MEKC system was successfully applied to the determination of related substances in MK bulk drug and its quantification in two pediatric pharmaceutical dosage forms.

Keywords:
Capillary electrophoresis / Cyclodextrins / Montelukast / Stereoselective analysis
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1 Introduction

Montelukast (MK) (sodium (R,E)-2-(1-(((1-(3-(2-(7-chloroquinolin-2-yl)vinyl)phenyl)-3-(2-(2-hydroxypropan-2-yl)phenyl)propyl)thio)methyl)cyclopropyl)acetate) is a potent and selective leukotriene antagonist receptor used in treatment of chronic asthma and seasonal allergic rhinitis. MK blocks $CysLT_1$, in lungs and bronchial tubes, reducing bronchoconstriction. It was the first leukotriene antagonist receptor granted for its use in children by FDA and it has been widely employed in the last years [1–5].

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Abbreviation: MK, Montelukast

MK production has been described by several synthetic processes involving a stereoselective synthesis [2, 3, 6]. Thus, MK has a chiral center in the carbon next to the thio side chain, and a double bound, which define both optical and geometric isomers, giving place to four stereoisomeric forms. Configuration of MK corresponds to *(R)-trans* isomer (Fig. 1A).

MK, like any compound obtained by chemical synthesis, could have substances or impurities that may originate from many sources (e.g. unreacted material, side reactions products, and/or degradation products).

(S)-trans isomer (Fig. 1B), an enantiomeric form of MK, is a by-product of the stereoselective synthesis, therefore the importance of the determination of MK enantiomeric purity [2, 6].

MK is also prone to chemical degradation as broadly described but many research groups such as Halama et al. [2] who clearly describe the functional groups and the type of degradation associate to them (Fig. 2). Most important

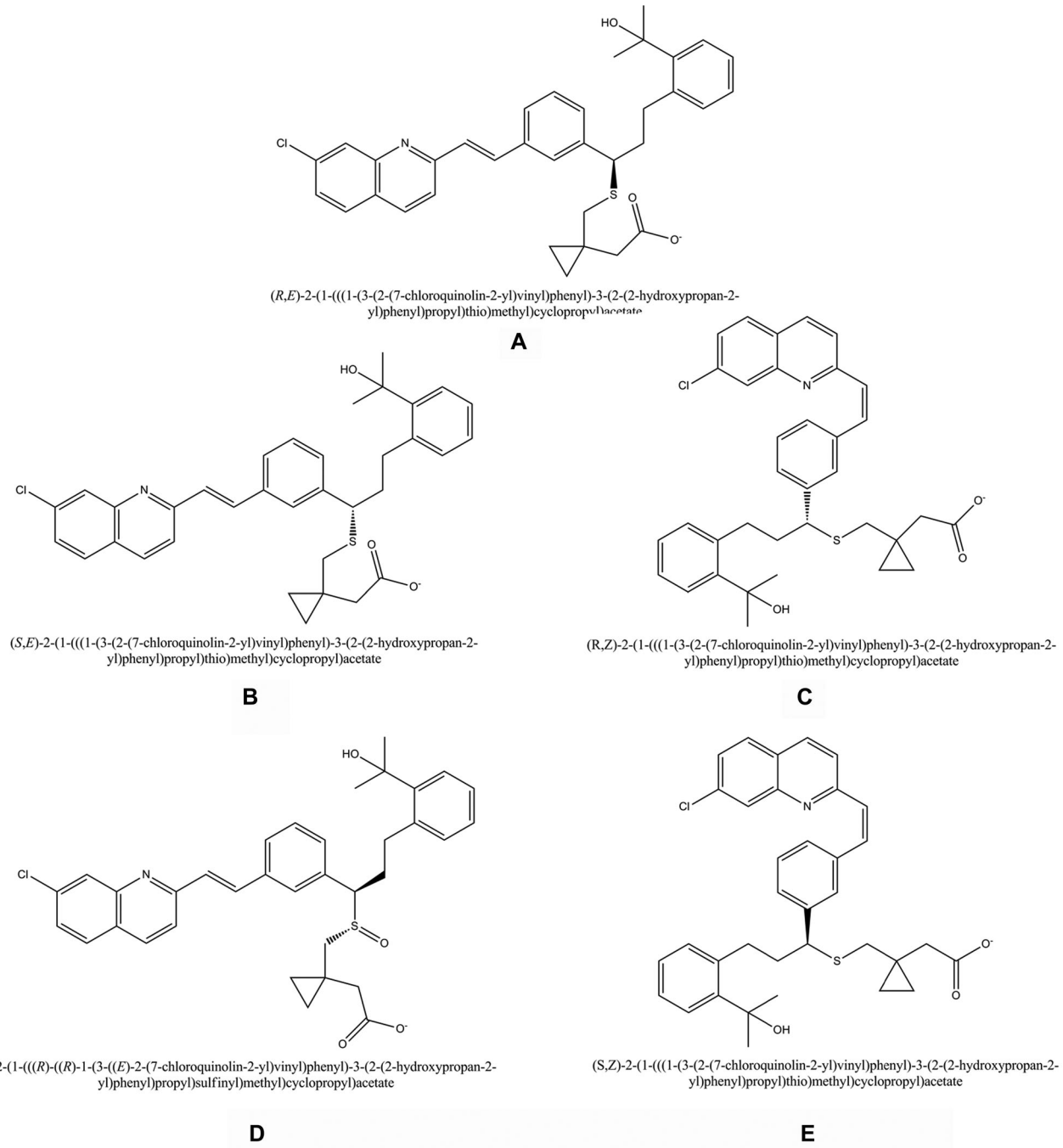

Figure 1. Chemical structure and IUPAC name of MK and main related substances.

degradation paths are photoisomerization (1), photooxidation (2), dehydration (3), and dehalogenation (4).

Photoisomerization and photooxidation are the main degradation pathways, and it is related to MK high sensitivity to light and moisture. Light exposure causes the rotation from *trans* configuration to the *cis* geometric configuration, leading to the formation of the *(R)-cis* isomer (Fig. 1C). *(S)-trans* isomer also suffers this configurational change to *(S)-cis* isomer, but given the low amounts of *(S)-trans* isomer in the samples, the concentration of *(S)-cis* isomer is insignificant. Therefore, upon its exposure to light and oxygen, but with slower kinetics, the mercapto group is oxidized leading to the formation of MK sulphoxide (MK-S-oxide) (Fig. 1D) [2,4,5,7]. So far no pharmacological and toxicological effects have been reported for *(S)-trans* isomer, *(R)-cis* isomer or MK-S-oxide.

MK monograph has been recently included in official pharmacopoeias such as USP and European Pharmacopoeia [8, 9]. In both official monographs, the proposed HPLC

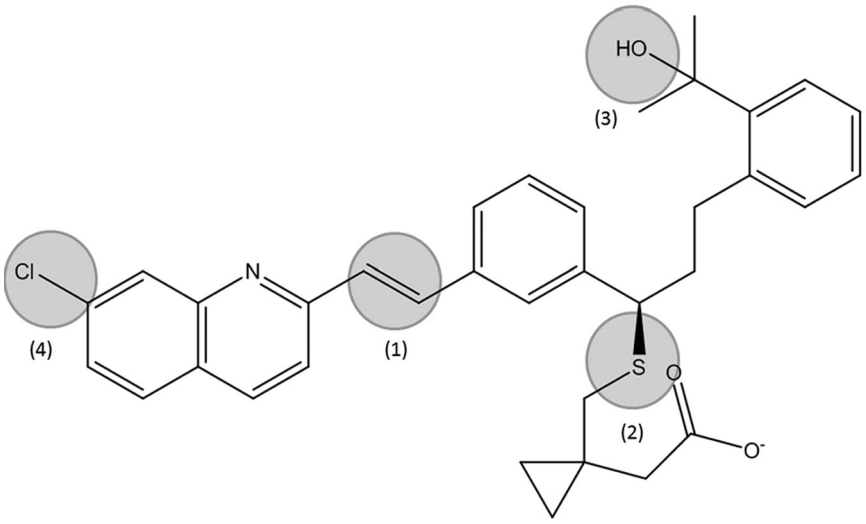

Figure 2. Functional groups of MK most susceptible to degradation.

method for the analysis of MK and related substances (sulfoxide impurity, *cis* isomer) uses a phenyl stationary phase, TFA, and ACN in the mobile phase and a gradient system, whereas enantiomeric purity test (for determination of S-enantiomer) requires a stationary phase with α1-acid glycoprotein as chiral selector and also a gradient system. The acceptance criteria is no more than 0.2% to sulfoxide impurity, 0.1% for *(R)-cis* isomer and 0.2% for *(S)-trans* isomer.

Moreover, several methods for the quantification of MK in pharmaceutical dosage forms, stability and pharmacokinetics studies have been developed. Most of them describe HPLC methods coupled to UV or MS detector [1,2,5,7,10,11]. Nevertheless, so far only one capillary electrophoretic method has been reported [4]. Although this method was able to separate MK from its *(R)-cis* isomer, is not applicable to MK separation from its enantiomeric form and did not identify its other main related substances such as MK-S-oxide for further quantification.

CE in its different modes such as CZE and EKC has been widely used as a powerful tool for enantioseparation in pharmaceutical analysis given to its high efficiency and resolution, low solvent consumption, and short analysis time, becoming an alternative to the conventional HPLC method [12–14]. Among all chiral selectors in CE, CDs are considered the most important mainly to their usefulness in the development of analytical separation methods and also for the study and characterization of supramolecular complex formation. Its utility in the enantioseparation of active pharmaceutical ingredients has been widely reviewed [15–17].

The aim of this work was to develop the first CD-MEKC system applied to the simultaneous determination of MK, its chiral impurity, its *cis* isomer and MK-S-oxide to be applied to the quality control of active pharmaceutical ingredients and different pharmaceutical dosage forms, combining univariate and multivariate designs during optimization steps. As far as we know, there are no reports of the simultaneous separation of these analytes by CE.

2 Materials and methods

2.1 Chemicals and reagents

Sodium MK (sodium (R,E)-2-(1-(((1-(3-(2-(7-chloroquinolin-2-yl)vinyl)phenyl)-3-(2-(2-hydroxypropan-2-yl)phenyl)propyl)thio)methyl)cyclopropyl)acetate (LOT F0J082) and MK racemate RS (LOT F0J344 MK RS) USP references standards (USP Rockville, MD) were gently supplied by Richet Laboratories (Argentina). SDS, sulfobutylether β-CD (substitution degree 6.8), (2,3,6-tri-O-methyl) β-CD, sulfated β-CD (substitution degree 12–15), 2-hydroxypropyl-γ-CD and sodium tetraborate salt, were purchased from Sigma (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Water was purified in an EASY Pure RF equipment (Barnstead, Dubuque, IA, USA).

2.2 Instruments

All CE separations were performed using a P/ACE MDQ CE system (Beckman, Fullerton, CA, USA). Uncoated fused silica capillaries (Microsolv technology, Eatontown, NJ, USA) of 60 cm (50 cm length to detector) × 75 μm id, were used. The separation was performed by a CD-MEKC system consisting of 10 mM SDS, 10 mM sulfobutylether-β-CD, 10 mM (2,3,6-tri-*O*-methyl) β-CD, and 20 mM borate buffer at pH 9.0. Separation was carried out under positive polarity at a constant voltage of 18 kV and capillary temperature was kept at 15°C. PDA detector range was 190–300 nm, while detection was set at 254 nm. Hydrodynamic injection was set at 0.5 psi for 5 s. A new capillary was conditioned by rinsing with 0.5 M potassium hydroxide for 3 min, 0.1 M potassium hydroxide for 2 min, and water during 2 min. Before each analysis, the capillary was washed with 0.1 M potassium hydroxide, water, and the BGE during 2 min for each one.

2.3 Standard and sample preparation

2.3.1 Stock and standard solution

2.3.1.1 Sodium MK and MK racemate (MK RS)

Stock solutions of MK at 1 mg/mL and MK RS at 2 mg/mL were prepared in methanol. Standard solutions of 50 and 100 μg/mL (1:1 of each enantiomer), respectively, were prepared by appropriate dilution of stock solution in dilution solvent (2 mM borate buffer).

2.3.1.2 *Cis* isomers

A portion of the stock solutions of MK and MK RS were divided into two separated glass vials and irradiated with UV light at 254 nm for 12 h.

2.3.1.3 MK sulfoxide (MK-S-oxide)

MK-S-oxide was synthetized according to a previously reported method by Halama et al. [2] and identified by Infrared and MS. A stock solution containing 1 mg/mL in methanol was prepared, and appropriately filled up with solvent diluent.

2.3.2 Sample preparation

2.3.2.1 Bulk drug

For the analysis of related substances, approximately 125 mg of bulk drug was accurately weighed into a 25.0 mL volumetric flask and 5.0 mL of methanol were added. The solution was sonicated until complete dissolution and then filled up with solvent diluent to a final volume (concentration around 5.0 mg/mL). On the other hand, for MK quantification around 32.0 mg of bulk drug was accurately weighed into 25.0 mL volumetric flask and filled up with methanol. One milliliter of this solution was then placed into a 25 mL volumetric flask, 4.0 mL of methanol added, and finally filled up with diluent to final volume.

2.3.2.2 Chewable tablets

Ten chewable tablets (MK label content 5 mg/tablets) were finely powered, and around 500 mg of this powder was accurately weighed into a 25.0 mL volumetric flask. Ten milliliters of methanol was added to the volumetric flask, and the dispersion sonicated for 20 min, filled up to final volume and subsequently centrifuged. Five milliliters of the supernatant was filled up with solvent diluent to a final volume of 50.0 mL into a volumetric flask.

2.3.2.3 Oral granules (pouches)

The content of five pouches (label content 4 mg of MK/pouch) was accurately placed into a 25.0 mL volumetric flask. Then, the samples were filled up like was previously described for chewable tablet.

3 Results and discussion

3.1 Method development and optimization

In order to carry out the MK separation, its enantiomer ((S)-*trans* isomer), their *cis* R,S diasteroisomeric forms and its main degradation product (MK-S-oxide), several parameters such as type and concentration of CD, buffer pH and concentration, voltage and temperature were evaluated. As initial system, a CD-MEKC for separation of MK and its *cis* isomer reported by Shakalisava et.al. was chosen [4]. The CD-MEKC consisted of 10 mM SDS, 10 mM 2-hydroxypropyl-γ-CD (2HP-γ-CD), and 20 mM borate buffer.

First, the influence of SDS was assessed. No resolution between MK and its (R)-*cis* isomer was obtained without SDS. This is evidence that system selectivity is given by the differential mobilities between the complexed forms (with the SDS and the CD) and not by the difference in mobilities between the free and the complexed form analyte CD. Thus, SDS concentration was increased from 5 to 50 mM. Resolution was improved with higher SDS concentrations, but analysis time was enlarged and current instability was pronounced. Therefore, 10 mM was chosen as the appropriate concentration of SDS.

Multiple CD systems have been widely used as these combinations provide increased enantioselectivity. In spite of the bibliography on the subject, it is difficult to predict which CD will provide the best result as multiple variables affect enantioselectivity, even more when a combination is used. Therefore, selection is done on an experimental basis, that is why a series of neutral and charged CDs (native β-CD (β-CD), native γ-CD, sulfated β-CD (SO4- β-CD), (2,3,6-tri-O-methyl) β-CD (TM-β-CD) and sulfobutylether β-CD (SBE-β-CD) were assayed at 10 mM as a second chiral selector apart from HP β-CD. Only with SBE-β-CD a slight separation was observed. Based on this result, different concentrations of SBE-β-CD were tested (5, 10, 12.5, 15 and 20 mM). Baseline resolution between MK and its enantiomer ((S)-*trans* isomer) was achieved at 12.5 mM. However, when a standard solution of MK, (S)-*trans* isomer, and (R)-*cis* isomer was analyzed, (R)-*cis* isomer positioned between (S)-*trans* isomer and MK compromising resolution (Fig. 3A). When 10 mM of TM-β-CD was added to the system, mobility of (R)-*cis* isomer was modified while resolution between MK and (S)-*trans* isomer was not significantly different (Fig. 3B). Therefore, each CD was assayed alone and in different combinations (Fig. 3C–E). In most cases combination of a neutral and a charged CD with charged analytes as MK provides a higher resolution due the fact that both CDs contribute to enantioselectivity. Finally, the complete resolution between (S)-*trans* isomer, MK, and also (S)-*cis* isomer and (R)-*cis* isomer (R_s 5.35, 14.88 and 1.85, respectively) was achieved with a final composition of 10 mM

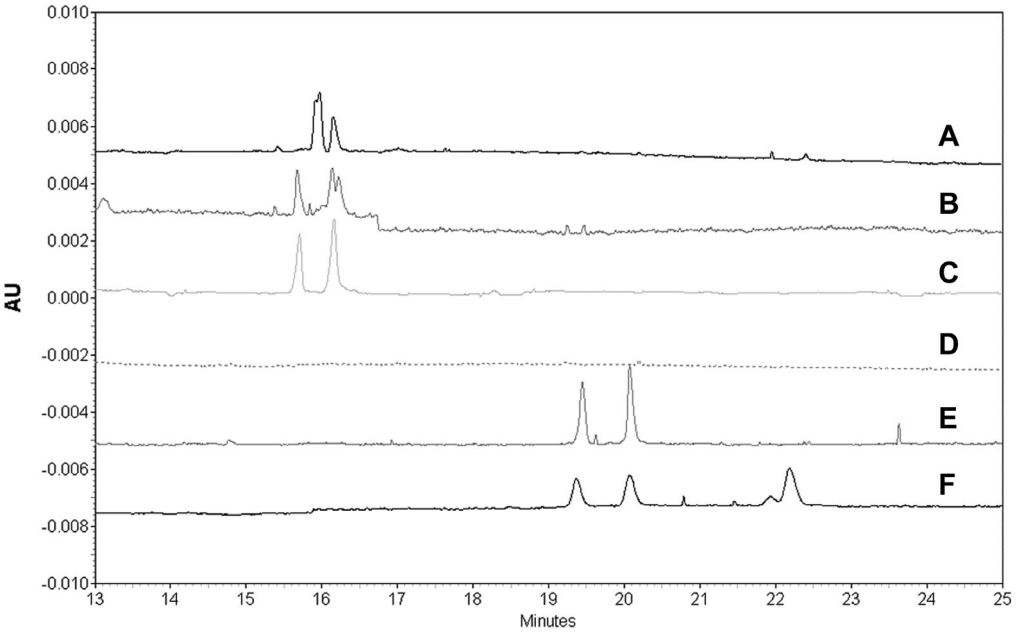

Figure 3. Electropherograms of CD type and concentration optimization. (1) *(S)-trans* isomer (2) *(R)-cis* isomer, *(3)* MK y (4) *(S)-cis* isomer. (A) 12.5 mM SBE-β-CD + 10 mM OH-γ-CD, (B) 12.5 mM SBE-β-CD +x 10 mM TM-β-CD, (C) 10 mM OH-γ-CD, (D) 12,5 mM SBE-β-CD, (E) 10 mM TM-β-CD, and (F) final conditions described in text.

SDS, 10 mM TM-β-CD, and 12.5 mM SBE-β-CD, and 20 mM borate buffer pH 9.0 (Fig. 3F).

Fixing the concentration of one CD, the influence of the other was analyzed (results are shown in Fig. 4). As can be seen, while increments of SBE-β-CD concentration in the assayed range does not significantly increases resolution (Fig. 4A), the concentration of TM-β-CD is crucial for the separation of the *cis* isomers pair from *trans* isomers (Fig. 4B).

Influence of buffer pH and voltage were also investigated. To optimize these two variables, multivariate optimization was carried out using a full factorial design with two factor three levels design (3^2) [18]. The measured response was resolution between MK and (S)-*trans* isomer. The main objective of using factorial design was to identify the conditions where an optimal resolution was achieved, reducing the number of experiments to nine. The pH levels chosen were 7.0, 9.0, and 11.0, while voltage levels were 12.0, 18.0, and 24.0 kV. Results are shown in an isoresponse plot (Fig. 4C). From the surface response plot, 18 kV and pH 9.0 were chosen as final electrophoretic conditions.

The influence of temperature in the electrophoretic mobilities was also evaluated between 15.0 and 35.0°C. Migration time was extended as temperature was increased. Therefore, 15°C was chosen for further analysis. DAD was used with a scan range from 190 to 300, and detection wavelength was set 254 with a 10 nm bandwidth.

After optimization, the final working conditions were as follows: BGE composed of 10 mM SDS, 10 mM TM-β-CD and 12.5 mM SBE-β-CD and 20 mM borate buffer pH 9.0, operational parameters 18 kV, 15°C, 254 nm detection wavelength (Fig. 5), remaining of the original composition only the SDS.

3.2 Validation

The developed method was validated according to ICH guidelines [19]. Parameters such as specificity, LOD and LOQ, linearity, precision, accuracy and robustness were tested. Specificity was demonstrated by three different assays. First MK was subjected to force degradation studies under different stress conditions (0.1 M NaOH and 0.1 M HCl under reflux for 1 h, 3% peroxide hydrogen and UV irradiation at 254 nm) and several degradation products were obtained, although none of them interfere with MK peak (Fig. 6). Also, a solution of MK (50 μg/mL) was spiked with known amounts of *(S)-trans* isomer, *cis* isomers, and MK-S-oxide. Also, excipients analysis was performed. Interfering peaks were not found under any of these conditions that demonstrated the high specificity of the method.

Linearity at five concentration levels with three replicates each, in a concentration range from 10 to 100 μg/mL were constructed in order to test linearity. Intercept and slope were 7346.6 and 15366.7, respectively, with a $R^2 = 0.9908$. LOD and LOQ were determined as 3 and 10 times S/N, respectively (Table 1).

Precision was evaluated for intraday ($n = 6$) and interday ($n = 18$) assays and it was expressed as RSD for peak areas and migration times. Accuracy was tested from recovery studies from two pharmaceutical matrixes (pouches and chewable tablets). Placebo samples emulating the excipients present in

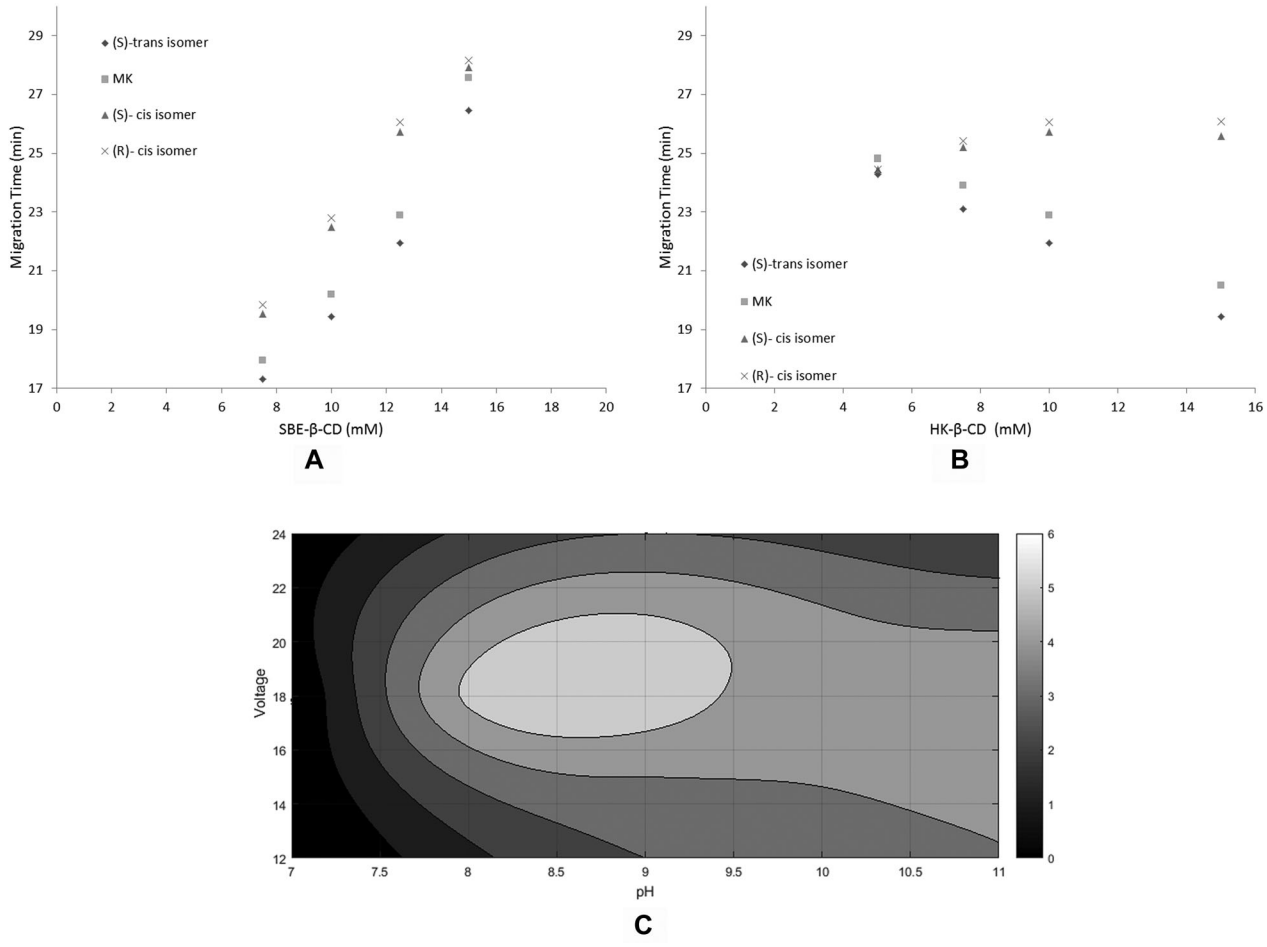

Figure 4. Migration times of the analytes versus A) increasing concentration of TM-β-CD (SBE-β-CD fixed 12.5 mM). (B) Increasing concentration of SBE-β-CD (HP-β-CD fixed 10 mM). (C) Surface response plot, voltage versus pH buffer.

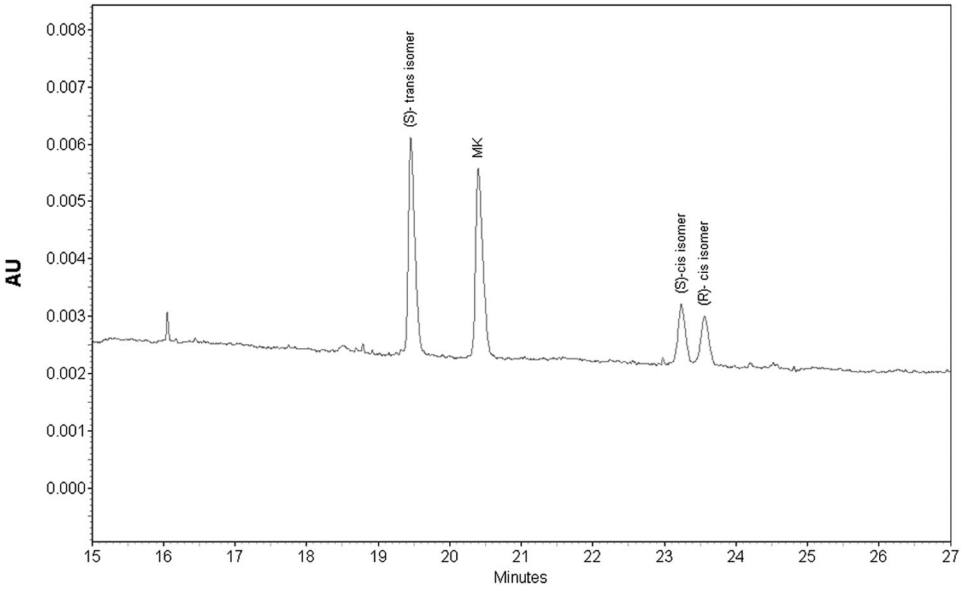

Figure 5. Separation of MK, (S)-*trans* isomer and *cis* isomers. Electrophoretic conditions described in text.

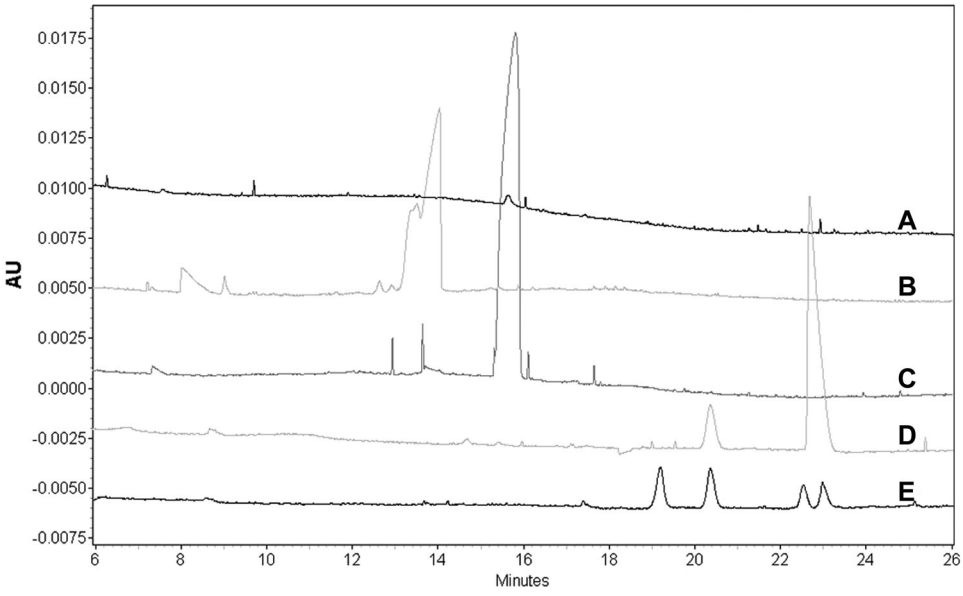

Figure 6. Electropherograms of stress assay. (A) 0.1 M HCl, (B) 3% peroxide hydrogen, (C) 0.1 M NaOH, (D) UV radiation at 254 nm and (E) standard solution of MK, its enantiomer and *cis* RS isomers.

Table 1. Validation parameters of MK

Parameter	MK		
Linear range (μg/mL)		10.0–100.0	
R^2		0.9908	
LOD (μg/mL)		0.30	
LOQ (μg/mL)		0.90	
Precision (%RSD)			
Intraday			
Peak area		1.4	
Migration time		0.7	
Interday			
Peak area		1.8	
Migration time		1.2	
Accuracy			
	80 %*	100%*	120%*
Chewable tablets**	100.3 (0.2)	99.0 (1.1)	101.9 (0.5)
Pouches**	99.8 (0.8)	100.9 (1.0)	101.2 (0.7)

*Respect to label content.
**RSD values between brackets corresponding to n = 3.
Linear range, LOD and LOQ for related compounds were also determined (Table 2).

Table 2. Linearity, LOD, and LOQ for MK related substances

Parameter	(S)-*trans* isomer	(R)-*cis* isomer	MK-S-oxide
Linear range (μg/mL)	1.00–20.0	1.00–20.0	5.00–20.0
R^2	0.9801	0.9915	0.9949
LOD (μg/mL)	0.30 (0.006)*	0.30 (0.006)*	1.70 (0.030)*
LOQ (μg/mL)	1.00 (0.020)*	1.00 (0.020)*	5.00 (0.100)*

*% w/w respect to a 5.0 mg/mL solution of MK.

the formulations were spiked with MK at concentration levels of 80, 100, and 120% of the nominal value. Three replicates of each level were assayed. Results are shown in Table 1.

For related substances linearity, LOD and LOQ were also determined. For *(S)-trans* isomer *and (R)-cis* isomer linearity was demonstrated within the range of 1.00–20.00 μg/mL (0.02–0.40% w/w respect to MK level of 5.0 mg/mL) and 1.00–20.00 μg/mL (0.10–0.40% w/w respect to MK 5.0 mg/mL) for MK-S-oxide, respectively (Table 2)

In order to study the robustness of the method a Plackett and Burman design with seven factors at two levels was applied. The seven variables included in the design were percentage of organic solvent in the injection (A), voltage (B), injection time (C), injection pressure (D), wavelength (E), operator (F), and day of analysis (G). The effects of the variables on the efficiency (N), tailing factor (T), mobility (μ), and resolution (R_s) (calculated according to USP [20]) were evaluated. Statistical analysis was used to determine the effect of each variable and its significance. To show the results, standardized Pareto plots were constructed. As can be seen in Fig. 7, none of the variables have a significant effect.

3.3 Real sample analysis

The developed method was applied to the analysis of related compounds and MK quantification in bulk drug and in two commercially available formulations (pouches and chewable tablets). Sample solutions were prepared according to the Section 2.3.2. Quantitation of MK and related compounds was performed under the conditions described in section 2.2. Results are described in Table 3.

4 Concluding remarks

The proposed method is the first one developed by CE for the simultaneous separation of MK, its enantiomer,

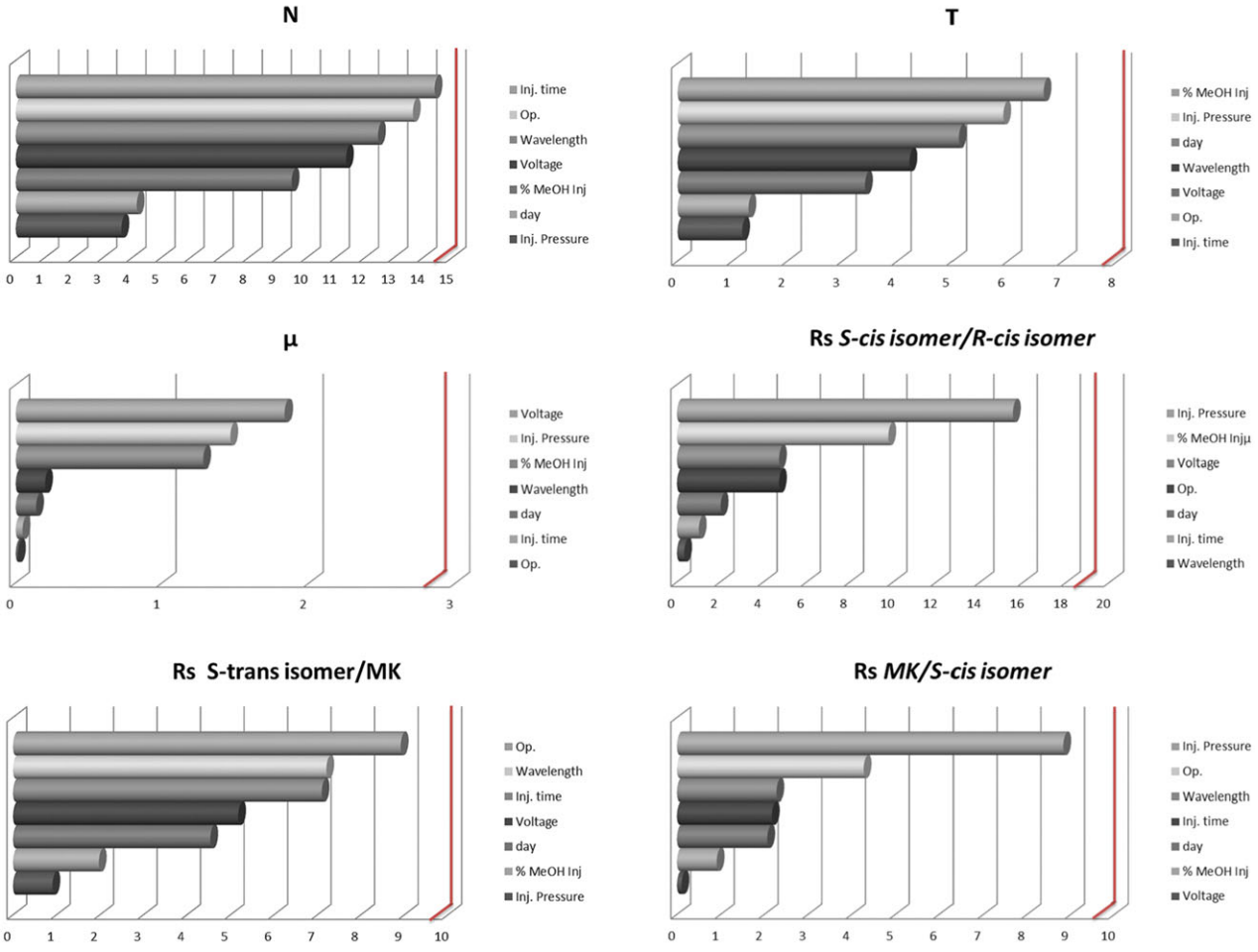

Figure 7. Pareto charts of standardized effects.

Table 3. Application of the CD-MEKC system on bulk drug and pharmaceutical formulations

	Found (mg/dose)	% Label
Bulk drug	99.5 (0.7)*	99.5
Pouches	5.26 (0.2)*	105.2
Chewable tablets	4.12 (1.0)*	103

*RSD values between brackets ($n = 3$).

diastereoisomer forms, and main degradation product. The combination of different CDs allows baseline separation with good resolution, among enantiomers, diastereoisomers and also MK-S-oxide. The method was validated according to ICH guidelines, based on the requirements of the official pharmacopoeia. The method has proved to be suitable for the quality control of bulk drug and in various pharmaceutical dosage forms.

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The authors have declared no conflict of interest.

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