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Rapid separation of tetracycline derivatives and their main degradation products by capillary zone electrophoresis

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A mixture of five tetracycline (TC) derivatives: minocycline (MC), demeclocycline (DMCTC), doxycycline (DC), and sancycline (SC), as well as each TC derivative from its main degradation product were separated by capillary zone electrophoresis (CZE). The influence of the pH and the concentration and nature of the background electrolyte (BGE) on the separations was investigated. Ethylenediaminetetraacetic acid (EDTA; 1 mM) was used as additive in a 25 mM phosphate buffer (pH 2.3) because this BGE enabled the rapid separation of the TC derivatives and of each TC derivative from its respective degradation product in less than 6 min. After optimization of the separation conditions, the analytical characteristics of the method were investigated. The parameters involved were linearity, precision (repeatability and reproducibility), and limits of detection (LODs). LODs obtained for the five TC derivatives studied were about 3 µg/mL. Finally, the CZE method developed was applied to study the stability of TC derivatives and to analyze the TC derivative content in three different pharmaceutical preparations.

Keywords: Tetracyclines / Method development / Optimization

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1 Introduction

Tetracyclines (TCs) are an important group of broad spectrum antibiotics widely used for both humans and animals [1]. TCs are structurally related compounds with multiple functional groups with acid-base properties, which presence confers them an amphoteric character. Most of these compounds exhibit an isoelectric point between 4 and 6. In acid solution and upon storage, TCs experience degradation reactions leading to products that are often isomers from the original precursor. The most important degradation products are those obtained by epimerization, dehydration, and combined epimerization-dehydration reactions [2]. On the other hand, commercial samples usually contain significant amounts of impurities (degradation products or different TC derivatives obtained in the synthesis) [3, 4] with only minor structural differences among them but differing widely in their pharmacological activities [1]. Therefore, analytical methods to perform the rapid analysis of TC antibiotics are needed in order to control their impurities in pharmaceutical preparations.

The physicochemical characteristics of TCs (ionogenic and water-soluble substances) make them suitable for electrophoretic analysis. Thus, in recent years different modes of capillary electrophoresis (CE) have been investigated to separate TCs and their impurities, such as capillary zone electrophoresis (CZE) [5–15], micellar electrokinetic chromatography [16, 17], nonaqueous CE [18–21], and capillary electrochromatography [6, 22]. CZE has been the most widely used mode of CE for separating TCs since in 1992 the first CE method for resolving TC from its degradation products was reported [5]. Although in this work an acid pH was used (pH 3.9), in general, CZE methods for the analysis of TCs employ buffers at basic pHs (from 8.5 to 12.25) in order to avoid sample adsorption on the capillary [8–15]. In these procedures, different kinds of additives such as ethylenediaminetetraacetic acid (EDTA), methanol, Triton X-100, or methyl-β-cyclodextrin (methyl-β-CD) were used to improve the separation selectivity, obtaining analysis times of about 15 min or larger.

Although different separations of TC derivatives have been performed, the separation of multicomponent mixtures has been scarcely reported and shorter analysis times are desirable in order to use analytical methodologies for routine analysis. In fact, as far as we know, only one CZE method has been reported on the separation of five TCs including TC, doxycycline (DC), and minocycline (MC) in about 8 min [6]. This method used a buffer at pH 3 with 40% methanol and β-alanine. The separation of four TCs (TC, DC, MC, and demeclocycline (DMCTC)) was not

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Abbreviations: DC, doxycycline; DMCTC, demeclocycline; MC, minocycline; SC, sancycline; TC, tetracycline

achieved using phosphate buffer at pH 7.5 because under these conditions three of them (MC, DC, and TC) were practically overlapped [7]. Also, the separation of DMCTC from MC or DC has only been performed using basic pH values with analysis times higher than 14 min [12–14]. On the other hand, some of the TCs have not been separated still from their degradation products. As an example, no reports have been found on the separation of sancycline (SC) from other TC derivatives or from its degradation products.

In this work, the rapid separation of five TCs (TC, MC, DMCTC, DC, and SC) and of each of these TCs and its main degradation product by CZE is presented. The influence of the pH, concentration and nature of the background electrolyte (BGE), and the presence of EDTA on the separations have been investigated. After optimization of the separation conditions, the analytical characteristics of the method have been investigated and a study of the stability of the five TCs has been performed.

2 Materials and methods

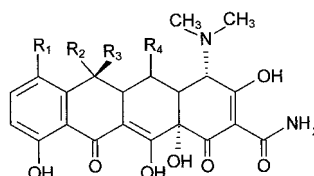
2.1 Apparatus

An HP^{3D} CE system (Hewlett-Packard, Waldbronn, Germany) equipped with an on-column diode array detector and an HP 3D-CE Chemstation software was used. Separations were performed on fused-silica capillaries of 50 μm ID and 375 μm OD, purchased from Composite Metal Services (Worcester, England). Capillaries had a total length of 33.5 cm and 25 cm to the detector. Capillary temperature was adjusted to 25°C. Finally, detection was performed at 265 nm. Electrolytic solutions were degassed in an ultrasonic bath KM from Raypa (Barcelona, Spain). A model 654 pH-meter from Metrohm (Herisau, Switzerland) was employed to adjust the pH of the BGEs.

2.2 Reagents and samples

All reagents employed were of analytical grade. Sodium dihydrogen phosphate dihydrate, trisodium phosphate dodecahydrate, dimethyl sulfoxide (DMSO), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were supplied from Merck (Darmstadt, Germany); disodium hydrogen phosphate dodecahydrate and EDTA disodium salt dihydrate were from Panreac (Barcelona, Spain); formic acid was obtained from Riedel-de Haën (Seelze, Germany); acetonitrile was from Lab Scan (Dublin, Ireland); water used to prepare solutions was purified through a Milli-Q system from Millipore (Bedford, MA, USA). Three

of the five TC antibiotics employed (DC, MC, and TC) were supplied by Sigma (St. Louis, MO, USA); DMCTC was from Fluka (Buchs, Switzerland), and SC was supplied by Hovione Sociedade Quimica (Lisbon, Portugal). Figure 1 shows the basic structure of these compounds. Three pharmaceutical preparations were analyzed in this work: MC in capsules, which contain MC (100 mg) with corn starch and magnesium stearate as excipients; DC in capsules, which contain DC with lactose, magnesium stearate, sodium lauryl sulfate, corn starch and alginate acid as excipients; DC in suspension, which contains DC with saccharin sodium, sodium hydroxide, calcium chloride, antifoam AF, apeline, eritrosine, carmine solution, sodium metabisulfite, butylparaben, propylparaben, polyvinyl pyrrolidone, veegum K, 70% sorbitol, glycerin, aroma of raspberry, and water. The three pharmaceutical preparations were acquired in chemists in Madrid (Spain).



Name	Symbol	R ₁	R ₂	R ₃	R ₄
Tetracycline	TC	H	CH ₃	OH	H
Minocycline	MC	N(CH ₃) ₂	H	H	H
Demeclocycline	DMCTC	Cl	OH	H	H
Doxycycline	DC	H	H	CH ₃	OH
Sancycline	SC	H	H	H	H

Figure 1. Chemical structures of the TC derivatives studied.

2.3 Procedure

Solutions of 200 mM formic acid or 100 mM phosphate buffer were prepared either by adding appropriate aliquots of concentrated formic acid or by dissolving the appropriate amount of phosphate salt into water. The different pHs for 100 mM phosphate buffers were adjusted as required by adding aliquots of 100 mM phosphoric acid solution. The concentrations 25, 50, and 75 mM, in phosphate buffer were obtained by diluting the 100 mM solution with water. The pH desired for the diluted solutions was adjusted with 1 M or 1 M NaOH. All electrolyte solutions were filtered prior use through 0.45 μm pore size disposable nylon filters from Scientific Resources (Eatontown, NJ, USA). Standard solutions were prepared by dissolving each TC antibiotic in DMSO

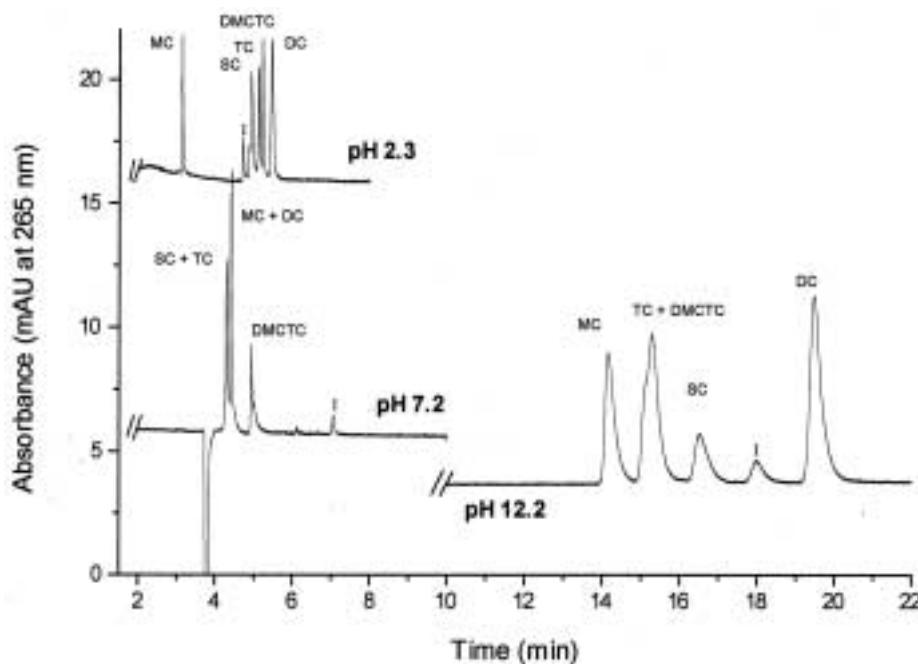


Figure 2. Separation of a mixture of the five TCs studied at acid, neutral, and basic pHs. BGE, 100 mM phosphate buffer; injection by pressure, 30 mbar for 2 s of sample followed by 30 mbar for 2 s of BGE; capillary, 33.5 cm (25 cm to the detector) \times 50 μ m ID; temperature, 25°C; applied voltage, 15 kV; current intensity, \sim 40 μ A; UV detection, at 265 nm. I, impurity.

to achieve the desired concentration. In order to obtain the degradation products of these compounds a solution of 0.1% phosphoric acid with acetonitrile (50:50, apparent pH 2.6) was employed. The solid products were dissolved in this solution and sonicated for 6 h before the analysis of the diluted solutions in water. Sample solutions for MC and DC were prepared dissolving in 10 mL of DMSO the total content of one capsule. These solutions (10 000 μ g/mL) and the suspension of DC (50 mg/5 mL = 10 000 μ g/mL) were diluted in DMSO in order to obtain a final concentration of 50 μ g/mL. These solutions were analyzed by the CZE method. Before first use, a new capillary was rinsed with 1 M NaOH for 30 min, 0.1 M HCl for 5 min followed by 30-min rinse with BGE. Between consecutive injections the capillary was only conditioned with the BGE for 4 min, but when the pH or the nature of the BGE was changed, before first injection a conditioning for 30 min with the new BGE was made. This conditioning method was used to obtain reproducible migration times. Injections were made by pressure: 30 mbar for 2 s of sample followed by 30 mbar for 2 s of BGE to improve the shape of the peaks.

3 Results and discussion

3.1 Method development

The separation of TC antibiotics can be highly influenced by the pH of the medium due to the fact that these substances are amphoteric compounds with isoelectric points ranging between 4 and 6. In a preliminary study,

we investigated the effect of pH using 100 mM phosphate buffer (acid, neutral, and basic pHs corresponding to the pK_a 's of the phosphoric acid, pH 2.3, 7.2, and 12.2). As shown in Fig. 2, the separation of a mixture of the five TCs was only possible at acid pH for which the best efficiency and the shortest analysis time were obtained. At pH 2.3 four concentrations of phosphate buffer were then considered (25, 50, 75, and 100 mM). A 25 mM phosphate buffer gave the lowest values of migration times and current intensity (\sim 40 μ A) without a significant loss in the resolution among the five TCs studied due to the small selectivity changes obtained when the concentration of BGE was increased. Therefore, a 25 mM phosphate buffer was selected. On the other hand, results showed that only with a 25 mM phosphate buffer a resolution higher than 1.5 (baseline resolution) was obtained for each TC derivative and its respective degradation product (the resolution between MC and its degradation product decreased when increasing the buffer concentration).

In order to improve the resolutions obtained, the influence of the nature of the BGE on the separation was investigated. Formic acid and phosphate buffer without or with EDTA were compared. Results showed that resolutions increased when 25 mM phosphate buffer (pH 2.3) instead of 200 mM formic acid (pH 2.2) was used. Moreover, the addition of 1 mM EDTA to 25 mM phosphate buffer enabled an improvement in the resolutions corresponding to the separation of TC and DMCTC and of each TC derivative and its respective degradation product.

Finally, a more exhaustive study on the influence of the pH (ranging from 2.3 to 5.3) was achieved when using 25 mM phosphate buffer with 1 mM EDTA. Table 1 shows the resolutions obtained for the separation of the five TCs studied at different acid pHs (from 2.3 to 4.3). It can be observed that only at pH 2.3 it was possible to obtain a baseline resolution of the five TCs whereas at pH 3.1, 3.7, or 4.3 only four of them were separated. On the other hand, at pH 5.3 no peaks were detected for tetracyclines because of their comigration with the electroosmotic flow (*i.e.*, with the peak of DMSO, which was the sample solvent). In addition, the analysis time increased when increasing pH values. Table 1 also shows that only at pH 2.3 it was possible to perform the separation of each TC derivative and its respective degradation product, while at pH 3.1 and 3.7 no resolution was observed for DC and its degradation product, and at pH 4.3 neither DC nor MC were resolved from their respective degradation products.

Table 1. Resolution obtained at different acid pHs for each two consecutive TCs in a mixture of the five studied and for each TC derivative and its main degradation product

pH	MC/SC	SC/TC	TC/ DMCTC	DMCTC/ DC	Analysis time (min)
2.3	24.3	1.8	1.3	2.7	5.5
3.1	25.2	0	1.8	7.9	7.9
3.7	45.3	1.7	0	6.3	10.6
4.3	31.5	1.2	0	3.3	12.5

pH	MC/D (MC)	SC/D (SC)	TC/D (TC)	DMCTC/D (DMCTC)	DC/D (DC)
2.3	3.7	3.6	4.2	4.6	1.2
3.1	10.2	8.4	15.7	14.2	0
3.7	5.5	8.2	22.9	15.6	0
4.3	0	5.6	11.6	11.6	0

BGE, 25 mM phosphate buffer-1 mM in EDTA. Experimental conditions: injection by pressure, 30 mbar for 2 s of sample followed by 30 mbar for 2 s of BGE; capillary, 33.5 cm (25 cm to the detector) \times 50 μ m ID; temperature, 25°C; applied voltage, 15 kV; current intensity, 40 μ A; UV detection, at 265 nm.

In conclusion, the study performed enabled to select 25 mM phosphate buffer (pH 2.3) with 1 mM EDTA as the most appropriate conditions for the rapid separation of TC derivatives studied and their degradation products. Under these selected conditions, the electropherogram of a mixture of the five TCs studied in this work has been obtained (Fig. 3). On the other hand, Fig. 4 shows the electropherograms obtained for the five TC derivatives

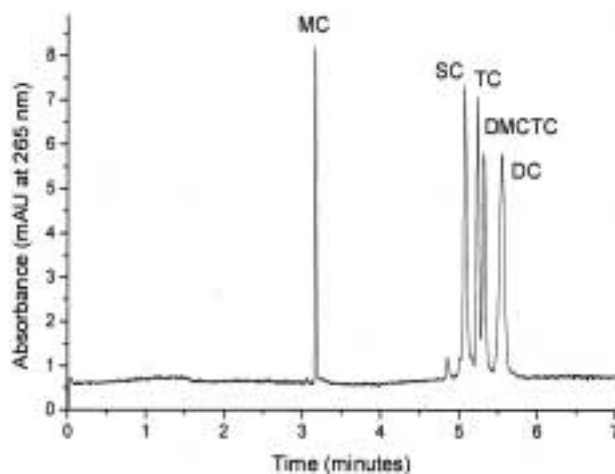


Figure 3. CZE of a mixture of five TCs. BGE, 25 mM phosphate buffer (pH 2.3) with 1 mM EDTA. Other conditions as in Fig. 2.

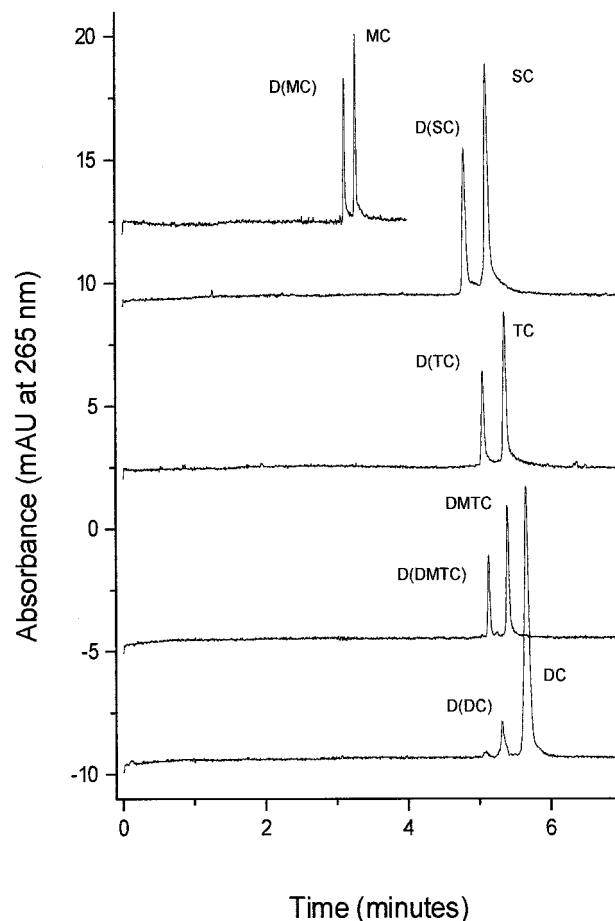


Figure 4. Separation of each TC derivative and its main degradation product by CZE. Experimental conditions as in Fig. 3.

Table 2. Analytic characteristics of the CZE method

	TC	MC	SC	DC	DMCTC
Calibration equation (Y = peak area; X = concentration of the TC derivate in $\mu\text{g/mL}$) six points ($n = 2$)	$Y = 0.770X - 5.294$ ($R^2 = 0.973$)	$Y = 0.246X + 0.181$ ($R^2 = 0.996$)	$Y = 0.587X + 1.288$ ($R^2 = 0.991$)	$Y = 1.065X - 6.671$ ($R^2 = 0.979$)	$Y = 0.416X + 1.626$ ($R^2 = 0.990$)
Repeatability ^{a)} ($n = 6$)	RSD (t_1) ^{d)} 0.9 RSD (t_2) 1.1 RSD (A_1) 1.9 RSD (A_2) 2.6 RSD (A_1/A_2) 1.2	1.2 1.3 1.9 1.3 0.9	0.2 0.7 2.8 0.6 2.7	1.3 1.5 2.0 1.0 2.0	2.4 2.6 4.2 2.0 2.2
Reproducibility ^{b)} ($n = 6$)	RSD (t_1) 0.7 RSD (t_2) 0.8 RSD (A_1/A_2) 1.7	2.0 2.2 4.1	0.5 0.6 2.2	2.1 2.3 7.2	0.7 0.8 5.4
LOD ($\mu\text{g/mL}$) ^{c)}	2.3	2.4	3.3	3.0	3.6

a) Measured from six consecutive injections of a solution of the same degraded TC derivative

b) Measured from two consecutive injections with three different capillaries

c) Calculated from the peak height based on a signal-to-noise ratio of 3

t_1 , migration time corresponding to the degradation product of the TC derivative, t_2 , migration time corresponding to the TC derivative, A_1 , peak area corresponding to the degradation product of the TC derivative, A_2 , peak area corresponding to the TC derivative, BGE, 25 mM phosphate buffer (pH 2.3)-1 mM EDTA, other experimental conditions as in Table 1.

d) in %

and their respective degradation products when the above-mentioned conditions were used. It can be observed that all TCs studied show a main degradation product, their epimeres. In order to show if the TC derivatives were stable during the time of the analysis, the percentage of degradation of each TC derivative studied, when dissolved in the 25 mM phosphate buffer at pH 2.3, was measured. Degradations lower than 3% were obtained after 60 min in phosphate buffer, therefore, the degradation of the TCs during the analysis by CZE (less than 6 min) is not significant.

3.2 Analytical characteristics of the method

After the development of the method for the rapid analysis of TCs, its analytical characteristics were examined, the results are shown in Table 2. The parameters involved were linearity, precision (repeatability and reproducibility), and limit of detection (LOD). The linearity study showed that linear relationships with good correlations in all cases ($R^2 > 0.99$) were obtained for the variation of the peak area or peak height as a function of the concentration of TC derivative (working range from 10 to 100 $\mu\text{g/mL}$), using at least six standard solutions (each solution was injected twice).

The precision of the CZE method described was measured as repeatability and reproducibility. Precision is

one of the most important criteria for evaluating the analytical method performance, and its numerical value is the relative standard deviation (RSD). The repeatability in the migration time, peak area, and ratio between the peak area of the degradation product generated and the peak area of the TC derivative (value proportional to % degradation) was determined as the RSD obtained for six consecutive injections of the degraded TC derivatives. Table 2 shows that acceptable levels of precision were obtained for the developed method in terms of repeatability (RSD _{$n=6$} from 0.2 to 2.6% for migration times, from 0.6 to 4.2% for peak area, and from 0.9 to 2.7% for the ratio between peak areas), since in all cases RSDs calculated were lower than 5%. The reproducibility of the method was measured as the RSD obtained for two consecutive injections with three different capillaries ($n = 6$). Acceptable RSD values were obtained for migration times (RSD < 2.5%), while only for DC a value of RSD < 2.5%), while only for DC a value of RSD \approx 7% was obtained for the ratio between peak areas (Table 2). Finally, an evaluation of the sensitivity was made by determining the values of the LOD calculated from the peak height based on a signal-to-noise ratio of 3. The noise was estimated as the largest deviation of detector signal from baseline measured in a section of about 5 min in the absence of analyte (1×10^{-4} AU). LODs for the five TC derivatives ranged from 2.3 to 3.6 $\mu\text{g/mL}$ (Table 2).

3.3 Application of the CZE method to study the stability of TCs and for the analysis of TC derivatives in pharmaceutical preparations

First, a stability study was performed measuring the degradation of the TCs dissolved in DMSO as an illustration of the applicability of the optimized CZE method for the analysis of TCs. The percentage of degradation of each TC derivative was calculated from the ratio between the peak area of the degradation product generated and the total peak area (the addition of the areas of the peaks of the TC derivative undegraded and its degradation product). The percentages of degradation obtained for TC, DMCTC, and SC were 3.6, 3.9, and 3.4%, respectively, when the solution was freshly prepared whereas for MC and DC peaks due to degradation products were not observed in these conditions. However, the degradation of these products was relatively fast (from 6.2% for DC to 12.4% for MC in 26 h). Finally, the percentages of degradation after five days were: 29.2% for TC, 29.0% for DMCTC, 17.5% for MC, 9.4% for SC, and 6.1% for DC. From these results it can be observed that only DC was quite stable after five days.

Second, the CZE method developed was applied to the analysis of MC and DC in three pharmaceutical preparations. Table 3 shows the content of these TC derivatives found by CZE in the three pharmaceutical preparations studied (two capsules and one suspension). The best results were obtained for the suspension of DC. The worst results were obtained for the capsules of DC and MC probably due to the difficulty in recovering the sample from the capsule. Good precision measured as RSD (%) was achieved for the measured concentrations (RSD values from 0.49% to 1.85%). Finally, Fig. 5 shows the electropherograms obtained for the three pharmaceutical preparations using the CZE method developed in this work. It can be emphasized that any interferent peak was observed.

Table 3. Content of two TC derivatives found by CZE in three pharmaceutical preparations

Sample	$C_{\text{label}}^{\text{a}}$	C_{CE}^{b} (RSD, %)
DC (suspension)	10 mg/mL	9.74 (0.49) mg/mL
DC (capsule)	100 mg	95.28 (1.85) mg
MC (capsule)	100 mg	91.32 (0.89) mg

a) C_{label} , concentration of the TC derivative specified on the label of the pharmaceutical preparation

b) C_{CE} , averaged concentration ($n = 5$) if the TC derivative content determined by the CZE method

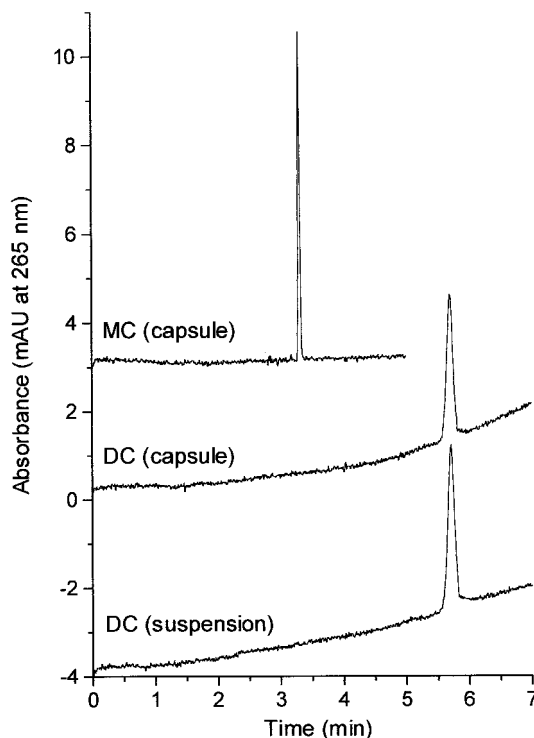


Figure 5. Analysis of MC and DC in three pharmaceutical preparations by CZE. Experimental conditions as in Fig. 3.

4 Concluding remarks

An analytical method has been developed to separate by CZE five TC derivatives (TC, MC, DMCTC, DC, and SC). A study on the influence of the pH, concentration and nature of the BGE, and the use of additives enabled to select 25 mM phosphate buffer (pH 2.3) with 1 mM EDTA in order to achieve two objectives: (i) the separation of a mixture of the five TC derivatives, and (ii) the separation of each TC derivative from its main degradation product (epimere). In both cases, the separations were performed in less than 6 min. The CZE method was evaluated by means of its linearity, precision (repeatability and reproducibility), and LODs. Good linearity was obtained for all the derivatives studied (working range from 10 to 100 $\mu\text{g/mL}$, $R^2 > 0.99$). The repeatability of migration times and peak areas measured as RSD was lower than 5%. The reproducibility of migration times and peak areas ratio between the epimere and the TC derivative using different capillaries was acceptable. LODs were about 3 $\mu\text{g/mL}$ for the five TCs studied.

Two applications of the CZE method were performed. A stability study of these compounds dissolved in DMSO was performed to check the reliability of the method employed. The results obtained enabled to conclude that when the solutions of these compounds are freshly pre-

pared, the method can be employed due to the short analysis time required for the analysis. In addition, the developed method was applied to the analysis of two of the TC derivatives studied (DC and MC) in pharmaceutical preparations.

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