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Microemulsion electrokinetic chromatography of corticosteroids Effect of surfactants and cyclodextrins on the separation selectivity

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Abstract

The separation of neutral hydrophobic corticosteroids (cortisone, cortisone acetate, hydrocortisone, hydrocortisone acetate, prednisolone and prednisolone acetate) by microemulsion electrokinetic chromatography (MEEKC) was studied. In the preparation of microemulsion, heptane was the solvent, *n*-butanol the co-surfactant and, as anionic surfactants, sodium dodecyl sulfate (SDS) or taurodeoxycholic acid sodium salt (STDC) were employed. Using an acidic running buffer, (phosphate pH 2.5) a strong suppression of the electroosmotic flow (EOF) was observed; this resulted in a fast anodic migration of the analytes partitioned into the negatively charged microemulsion droplets. Under these conditions, STDC showed better separation of corticosteroids than the conventional SDS; however, the use of a single anionic surfactant did not provide the required selectivity. The addition of the neutral surfactant polyoxyethylene glycol octadecyl ether (Brij 76) significantly altered the migration of each analytes allowing a better tuning of separation; however, in order to obtain adequate resolution between couples of adjacent critical peaks, the addition of neutral cyclodextrins (CDs) was found to be essential. This apparently complex system (CD-MEEKC), was optimized by studying the effect of the most important parameters affecting separation: STDC concentration, Brij 76 concentration, nature and concentration of cyclodextrins. Following a rational step-by-step approach, the optimised conditions providing the complete separation of the analytes were found to be: 4.0% STDC, 2.5% Brij 76, 6.6% *n*-butanol, 1.36% heptane and 85.54% of a solution 5 mM β-CD in 50 mM phosphate buffer (pH 2.5). The optimized system was preliminary applied to the detection of corticosteroids related substances at impurity level and it could be considered a useful orthogonal alternative to HPLC methods.
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Keywords: Microemulsion electrokinetic chromatography; Corticosteroids; Surfactants; Cyclodextrins; Related substances

1. Introduction

Capillary electrophoresis (CE), due to its various operation modes such as zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and microemulsion electrokinetic chromatography (MEEKC), has became a versatile alternative or complementary technique to the liquid chromatography (HPLC), useful in pharmaceutical analysis [1–4]. In particular, the MEEKC approach has recently gain great attention; its potential has been revised [5–7] and several applications to the analysis of drugs [8–12] and natural products [13–16] have been reported.

The MEEKC mode is based on the partitioning of neutral or charged analytes between moving charged oil droplets and the aqueous buffer phase. The oil-in-water (o/w) microemulsions are generally obtained by mixing oil (e.g. *n*-heptane) with water and by adding a surfactant such as sodium dodecyl sulfate (SDS) allowing negatively charged oil droplets to be formed. A co-surfactant (e.g. *n*-butanol) is also added to further stabilize the microemulsion.

As for the conventional MEKC, the migration of neutral analytes under MEEKC conditions is affected by the pH of the BGE due to the influence on the electroosmotic flow (EOF). In a fused-silica capillary, the presence of a substantial EOF (neutral or alkaline conditions) drives the negatively charged oil droplets towards the cathode; hydrophobic solutes, strongly retained by the anionic "pseudostationary phase" move toward the detector end slower than the water-soluble

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analytes. Differently, under strong acidic conditions the EOF can be greatly suppressed; as a consequence the negatively charged oil droplets will move toward the anode, allowing the partitioned analytes to be detected under reversed polarity. In such a configuration, labelled as reversed-flow (RF) condition, higher the hydrophobicity of the neutral solutes and faster is the migration. The role played by organic solvents on the mechanism of MEEKC separations has been reviewed [17]. Hansen et al. [18–20] showed the important effect of the surfactant and co-surfactant nature in controlling the analytes migration order. Previous studies in our laboratory showed the significant role of co-surfactant (aliphatic alcohol) in tuning the selectivity in the separation of hydrophilic compounds such as catechins [16]. Recently, strategies for optimisation of the MEEKC separation of drugs of varying charge and hydrophobicity have been reported [21,22].

The present study was undertaken to achieve further information for tuning the MEEKC selectivity in the analysis of highly hydrophobic analytes, such as corticosteroids. In particular, the optimum conditions were investigated to develop a selective method providing the separation of six structurally related corticosteroids: cortisone, cortisone acetate, hydrocortisone, hydrocortisone acetate, prednisolone and prednisolone acetate. These steroids are potential impurities one another and the European Pharmacopoeia (EP) prescribes reversed phase liquid chromatography (HPLC) for their quality control [23].

For this application, a MEEKC system, involving anionic surfactants at pH 2.5 was chosen. Under these conditions the EOF was greatly suppressed and the separation selectivity was enhanced by following integrated approaches: (a) use of alternative surfactants to the conventional SDS; (b) addition of a non-ionic surfactant to slow down the migration of the oil droplets; (c) addition of neutral cyclodextrins (CDs) to selectively retain the analytes in the aqueous solution by CD-inclusion complexation. The developed cyclodextrin-modified MEEKC (CD-MEEKC) method proved to be suitable to detect (UV absorption at 254 nm) related steroids at impurity level (0.5%, w/w) as required by European Pharmacopoeia.

2. Experimental

2.1. Chemicals

Cortisone, cortisone acetate, hydrocortisone, hydrocortisone acetate, prednisolone, prednisolone acetate, sodium taurodeoxycholate (STDC) were obtained from Sigma Italia (Milan). Sodium dodecyl sulfate (SDS), β-cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD), heptakis (2, 3, 6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD), polyoxyethylenesorbitan monooleate (Tween 80), 3-[(3-cholamidopropyl)dimethylammonium]-1-propansulfonate (CHAPS) and polyethylen glycol octadecyl ether (Brij 76) were from Fluka (Buchs, Switzerland). All the other chemicals were purchased from Carlo Erba Reagents (Milan, Italy). The water used for the preparation of solutions and running buffers was purified by a Milli-RX apparatus (Millipore, Milford, MA, USA).

Stock solutions of each corticosteroid (2 mg/ml) were prepared in methanol as well as the mixed standard solution containing all the steroids (0.33 mg/ml); this last solution was diluted 1:4 with the microemulsion used in the analysis (final concentration: 0.083 mg/ml). Solutions containing a reference standard steroid (2 mg/ml) as main drug in the presence of the related impurities 0.01 mg/ml (thus corresponding to the 0.5%, w/w) were prepared in methanol and were diluted 1:2 with the microemulsion before the injection.

2.2. Calibration graphs

Linearity was evaluated for hydrocortisone acetate and prednisolone which were selected as model compounds owing to their characteristic chromophores representative of all the studied corticosteroids. Stock solutions were properly diluted with a mixture of methanol: microemulsion 1:1 (v/v) in order to cover the calibration range of 0.005–0.08 mg/ml for both the analytes and in the presence of cortisone as internal standard (concentration of 0.09 mg/ml). After triplicate analysis under the optimised MEEKC conditions of each of the samples (five calibration points), the corrected peak area ratios (analyte to internal standard) were plotted against the corresponding concentration to obtain the calibration graphs.

2.3. CE apparatus and conditions

Electrophoretic analyses were carried out using a Biofocus 2000 system (Bio-Rad Hercules, CA, USA). The data were collected on a personal computer using a Biofocus System Integration Software Version 5.2. An untreated fused-silica capillary of total length 24 cm (effective length 19.5 cm) × 50 μm I.D. was used. All the separations were carried out at 40 °C with an optimised voltage maintained constant at 10 kV (reversed polarity); hydrodynamic injections were performed at 1 psi for 2 s and the detection wavelength was 254 nm.

Prior the first use, the capillary was conditioned by flushing sequentially: 1 M sodium hydroxide, 0.1 M sodium hydroxide and finally water (10 min each). The capillary was equilibrated (10 min) at the beginning of the day with the running buffer. The rinsing procedure was found to be very important to obtain reproducible migration times; the highest reproducibility was achieved by flushing the capillary between the runs as follows: 1 min with methanol and 3 min with the BGE. Vials of BGE were replaced every injection to avoid the electrolysis of the solutions.

2.4. Microemulsion preparation and characterization

The microemulsions were prepared by weighing each component (*n*-heptane, surfactant, co-surfactant, pH 2.5 buffer) at the appropriate amount according to specific experiments consisting in the adjustment of the proportion of constituents (titration) in order to assure high thermodynamic stability and UV transparency of the preparation. Every mixture was sonicated until the system turned clear (10–30 min) and finally, prior the use, the microemulsion was filtered through a 0.2 μm membrane (Chemtek, Bologna, Italy).

Dynamic light scattering measurements [16,24] were carried out to estimate the average droplets' size of microemulsions at different temperatures (20, 30 and 40 °C) using an instrument (Broookhaven 90-PLUS) equipped with a 50 mW He–Ne laser (532 nm). Measurements were performed as previously described [16]. The hydrodynamic diameter, $d_{\rm H}$, was calculated using the Stokes–Einstein equation:

$$d_{\rm H} = k_{\rm B}\frac{T}{3\pi\eta_0 D}$$

were $k_{\rm B}$, T, η_0 and D are the Boltzman constant, the absolute temperature, the solvent viscosity and the translational diffusion coefficient, respectively.

3. Results and discussion

3.1. Method development

Few studies concerning MEEKC separation of steroids have been reported [9,25,26]; these methods were generally applied to standard mixtures of steroids having significantly different structure. The corticosteroids considered in this study (Fig. 1) are structurally correlated and their resolution can offer a useful contribution to approach real analytical tasks.

Previously, acidic conditions (pH 2.5) were found to be convenient in MEEKC analysis of mixtures of catechins, natural antioxidant compounds unstable under alkaline conditions [16]. Aim of the present study was to verify the applicability of strong acidic (pH 2.5) MEEKC to the separation of highly hydrophobic compounds bearing ester groups and to find the conditions useful for tuning the separation selectivity. Under the described acidic conditions the EOF is greatly suppressed and the neutral studied analytes were subjected to a chromatographic-type separation mechanism: the resolution of the analytes is exclusively driven by their selective distribution between the negatively charged oil droplets moving to the anode (detector), and the aqueous phase. As a conse-

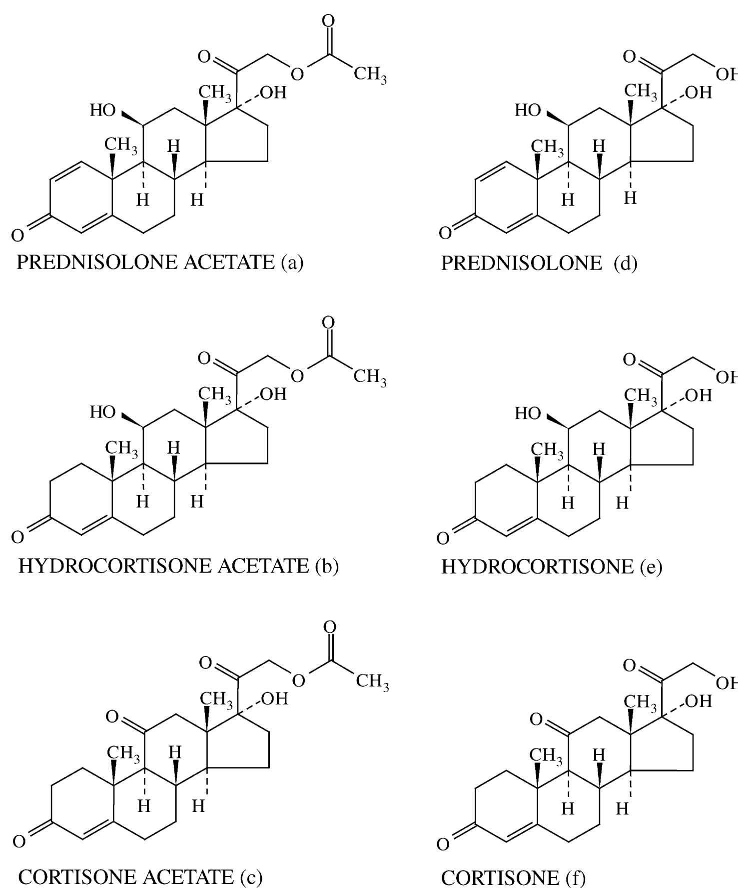

Fig. 1. Structure of the studied analytes.

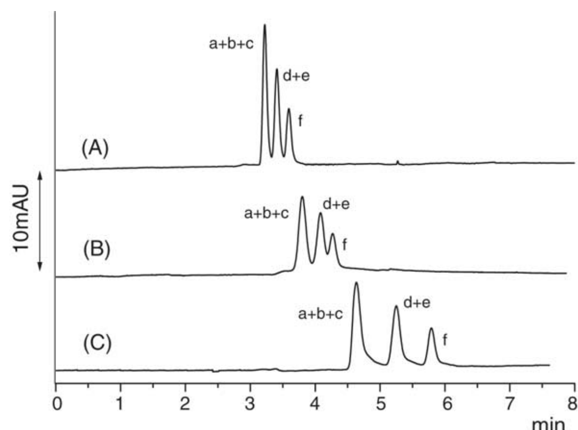

Fig. 2. Effect of SDS concentration on the separation of a test mixture of steroids under MEEKC conditions: 0.8% (w/v) *n*-heptane, 6.6% (w/v) *n*-butanol and: (A) 3.3% (w/v) SDS, 89.3% (w/v) 50 mM phosphate buffer pH 2.5. (B) 2.9% (w/v) SDS, 89.7% (w/v) 50 mM phosphate buffer pH 2.5. (C) 1.7% (w/v) SDS, 90.9% (w/v) 50 mM phosphate buffer pH 2.5. Other conditions: fused-silica capillary 24 cm (19.5 cm length to the detector); hydrodynamic injection at 1 psi × 2 s; voltage 10 kV (anodic detection at 254 nm); temperature 40 °C. Symbols: (a) prednisolone acetate; (b) hydrocortisone acetate; (c) cortisone acetate; (d) prednisolone; (e) hydrocortisone and (f) cortisone.

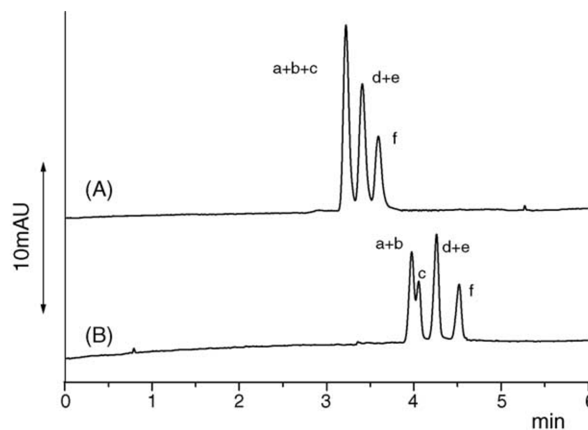

Fig. 3. Electropherograms of the separation of a text mixture of steroids under MEEKC conditions involving two different anionic surfactants. Conditions: 1.36% (w/v) *n*-heptane, 6.6% (w/v) *n*-butanol in 50 mM phosphate running buffer pH 2.5 and: (A) 3.3% (w/v) SDS; (B) 4.0% STDC. Other conditions and symbols as in Fig. 2.

quence, under these reversed-flow conditions, the neutral solutes strongly partitioned into the oil droplets (lipophilic compounds) migrate faster than the less partitioned compounds (hydrophilic solutes).

3.1.1. *Use of mixed -microemulsions*

Preliminary experiments were performed using standard MEEKC conditions: 0.8% (w/v) *n*-heptane, 3.3% (w/v) SDS, 6.6% (w/v) *n*-butanol and 89.3% (w/v) pH 2.5 phosphate buffer (50 mM). As shown in Fig. 2A, poor selectivity was obtained; the steroid acetates (labelled as a, b and c) migrated rapidly as a single peak (with a migration time of 3.2 min) according to their high hydrophobicity. Variation of the SDS concentration affects the partition of the analytes as well as migration velocity by changing the charge density of the oil droplets; in particular, under the so-called reversed flow microemulsion electrokinetic chromatography (RF-MEEKC), the decrease of SDS concentration resulted in increased migration times without improving the resolution between steroids of comparable lipophilicity (Fig. 2). As widely reported, also the nature of the surfactant should play a key-role in the partition control of hydrophobic solutes [19]; thus the use of an alternative anionic surfactant in substitution to SDS can be useful to improve the separation of the studied steroids. Due to the strong acidic pH of running buffer, the choice of the surfactant was restricted to the compounds bearing a sulfate group, which results completely dissociated at the chosen pH value. Tauro-conjugated bile acid salts, possess this key-feature; furthermore, their structural similitude (steroid skeleton) with the studied analytes, makes them attractive as an alternative to conventional SDS. On the other hand, bile acids proved to be suitable for MEKC separation of steroids [25–27]. Therefore, taurodeoxycholic acid sodium salt (STDC) was used; the amount of STDC in the preparation was evaluated by means of systematic addition of the surfactant (titration) to the mixture of the components till a stable and transparent microemulsion was obtained. Using 4.0% (w/v) of STDC a slightly improved separation was obtained in comparison to that using SDS (Fig. 3) although similar short migration times were obtained. In order to further improve the resolution, the choice of STDC was integrated with an additional approach directed to increase the migration times through the lowering of droplets charge density. In particular, a second neutral surfactant was supplemented to STDC to give preparations which can be labelled as mixed-MEEKC. Namely, Tween 80, Brij 76 (non-ionic surfactants) and CHAPS (zwitterionic surfactant) were individually tried by supplementing each of them to STDC with the aim to slow down the anodic migration of the "pseudostationary phase" without significant changes in the phase ratio.

Among the three additional surfactants, Brij 76 exhibited the best behaviour; in fact, in the presence of this additional surfactant the amount of STDC was reduced without disruption of microemulsion. Using 1.0% of Brij 76, a clear and stable microemulsion was obtained in the presence of 2.5% STDC; thus, the effect of Brij 76 on the separation was studied in the range 1.0–2.5% (w/v) fixing STDC at 2.5%. As expected and shown in Fig. 4 the increase of Brij 76 concentration led to a progressive and general increase of migration times; furthermore, since this behaviour seems to be followed for each of the solutes at the same extent, the observed improve in resolution should be ascribed to the slackening of oil droplets. These considerations support the choice of high percentages of neutral surfactant to obtain complete separation; however levels of Brij 76 higher than 2.5% were not useful resulting in long analysis times. Thus, by fixing the Brij 76 at 2.5%, the effect of STDC was evaluated; as shown in Fig. 5 the best result was obtained in the presence of 4.0% (w/v)

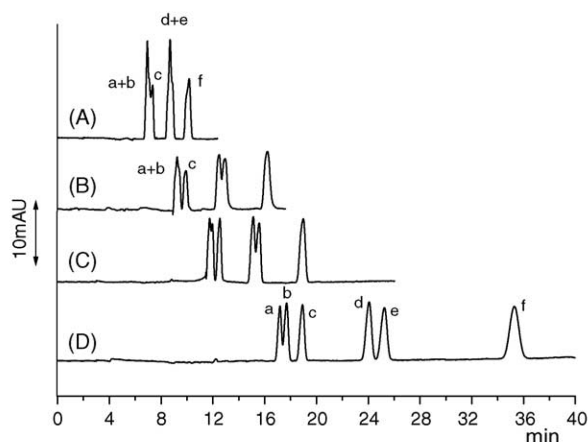

Fig. 4. Effect of Brij 76 concentration on the separation of a test mixture of steroids under MEEKC conditions: 1.36% (w/v) *n*-heptane, 6.6% (w/v) *n*-butanol, 2.5% (w/v) STDC, 50 mM phosphate running buffer and Brij 76 at: (A) 1.0% (w/v), (B) 1.5% (w/v), (C) 2.0% (w/v) and (D) 2.5% (w/v). Other conditions and symbols as in Fig. 2.

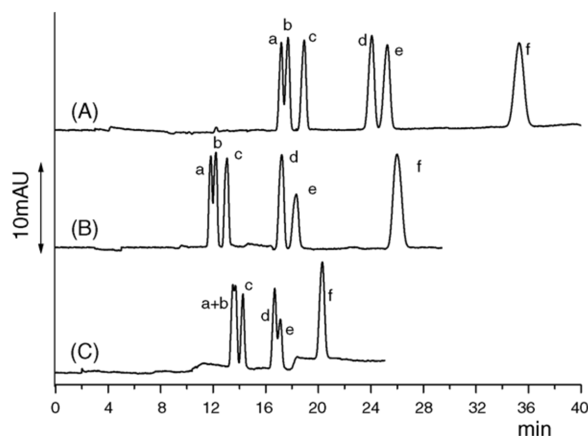

Fig. 5. Effect of STDC concentration on the separation of a test mixture of steroids under MEEKC conditions: 1.36% (w/v) *n*-heptane, 6.6% (w/v) *n*-butanol, 2.5% (w/v) Brij 76, 50 mM phosphate running buffer and STDC at: (A) 2.5% (w/v), (B) 4.0% (w/v) and (C) 5.0% (w/v). Other conditions and symbols as in Fig. 2.

of STDC since higher concentrations provided a loss of separation. These conditions (4.0% STDC, 2.5% Brij 76, 6.6% *n*-butanol, 1.36% *n*-heptane and 85.54% phosphate buffer pH 2.5) provided the complete separation of four of the six steroids in a reasonable analysis time; thus, this final preparation was subjected to dynamic light scattering experiments in order to estimate the average droplets' size. The results of these experiments are reported in Table 1; from these data the character of microemulsion can be confirmed since the droplets' size resulted within the typical range of microemulsion ("swollen micelles") and it was poorly affected by temperature.

Table 1
Characteristics of the optimized microemulsion

Temperature (°C)	Diameter (nm)	Polydispersity index
20	5.5	0.196
30	5.2	0.208
40	5.4	0.202

4.0% (w/v) STDC, 2.5% (w/v) Brij 76, 6.6% (w/v) *n*-butanol, 1.36% (w/v) *n*-heptane and 85.54% (w/v) phosphate buffer pH 2.5.

3.1.2. Use of cyclodextrins in MEEKC: cyclodextrin-modified MEEKC (CD-MEEKC)

Due to the necessity to increase the separation of the couple of adjacent and partially co-migrating peaks prednisolone acetate and hydrocortisone acetate (a and b), a further variable was introduced in the MEEKC system. Precisely, neutral CDs were chosen as an additional component of the separation mixture due to their ability in inclusion complexation of a number of drugs. In our system, the CDs could be though completely distributed into the aqueous phase of the bulk solution to give a second "pseudostationary phase" which acts in competition with the anionic "swollen micelles". In the absence of a significant EOF, the neutral CDs substantially create a not moving environment toward which the analytes transported by the microemulsion could be included and slackened. On the base of this rational, different neutral CDs were added to the previously optimised system; the electropherograms obtained by using a 5 mM concentration of each CD are shown in Fig. 6. As can be seen, with the exception of TM-β-CD, a complete separation of the analytes is obtained; on the base of this observation the hypothesized mechanism of inclusion complexation of the analytes into the cavity of not moving CDs resulted to be favourable. The separation profile however seemed to be not affected by the nature of CD; actually, only slight difference in migration time can be observed for cortisone (f), whereas all the other drugs showed almost the same migration time using the different CDs. For routine analysis β-CD was chosen as useful additive to obtain the complete separation.

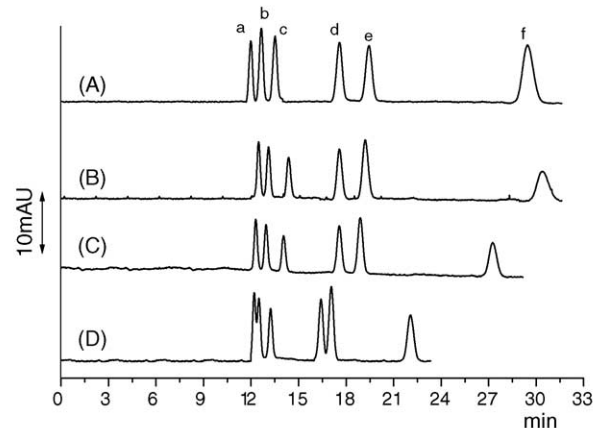

Fig. 6. Effect of the nature of cyclodextrin (5 mM) on the separation of a test mixture of steroids under MEEKC conditions: 1.36% (w/v) *n*-heptane, 6.6% (w/v) *n*-butanol, 2.5% (w/v) Brij 76, 4.0% (w/v) STDC, 85.54% (w/v) 50 mM phosphate running buffer pH 2.5. (A) β-CD, (B) HP-β-CD, (C) DM-β-CD and (D) TM-β-CD. Other conditions and symbols as in Fig. 2.

3.2. Detection of related substances in corticosteroids

The final optimized method consisting in CD-MEEKC (4.0% STDC, 2.5% Brij 76, 6.6% *n*-butanol, 1.36% *n*-heptane and 85.54% of phosphate buffer pH 2.5 containing 5 mM of β-CD) showed preliminary reliability and it satisfy some important requirements to encourage future validation approach. In particular, the stability of migration times was adequate as proved by intra-day RSD < 1.52%, $n = 5$ and inter-day repeatability RSD < 4.25%, $n = 9$.

The linearity of response to detector was evaluated for two of the analysed corticosteroids, namely hydrocortisone acetate and prednisolone, as representative of the two different chromophores of all the studied compounds. In the presence of cortisone as internal standard the corrected peak area ratios (analyte versus internal standard; *Y*) were plotted against the concentration (*C*) varying within a typical impurity range for each of the analytes (0.005–0.08 mg/ml). After regression analysis the following equations were obtained respectively for hydrocortisone and prednisolone:

$$Y = 0.07222(\pm 0.04639) + 15.38234(\pm 0.77387)\,C;\; r = 0.998.$$

$$Y = 0.02566(\pm 0.03631) + 16.721(\pm 0.55764)\,C;\; r = 0.999.$$

The sensitivity expressed as LOD was evaluated by progressive dilution of standard solutions of hydrocortisone acetate and prednisolone till a signal to noise (*S*/*N*) ratio of 3 was obtained; at the detection wavelength of 254 nm, these limits were in the order of 1.5 μg/ml for both the analytes. The lowest level of calibration range was reasonably assumed as limit of quantitation (RSD < 3.5%; $n = 5$).

The ability of the method to detect potential impurities was evaluated by analysing artificial mixtures of the main drug in the presence of the related substance(s) at 0.5% (w/w) level, as recommended by EP. Precisely, hydrocortisone (main drug) was completely separated by its related substances hydrocortisone acetate, prednisolone and cortisone (Fig. 7A); under the same MEEKC conditions prednisolone was resolved from hydrocortisone (Fig. 7B). In analogy, cortisone acetate was resolved from hydrocortisone acetate at impurity level of 0.5% (Fig. 8A) as well as prednisolone acetate was resolved from the impurities hydrocortisone acetate and prednisolone (Fig. 8B). In these last two analyses, the separations were carried out at 30 °C in order to better resolve the peaks with so big differences in concentration. The percentage ratios between the corrected peaks area (a) of main drug and related substances (%, a/a) were found to be in agreement with the mass percentage ratios (w/w), as suggested by the similitude of UV absorption of the analytes. The reliability of these data was underlined by the repeatability of corrected peak area determined at the impurity levels; intra-day values corresponding to RSD < 3.5% ($n = 3$) were obtained.

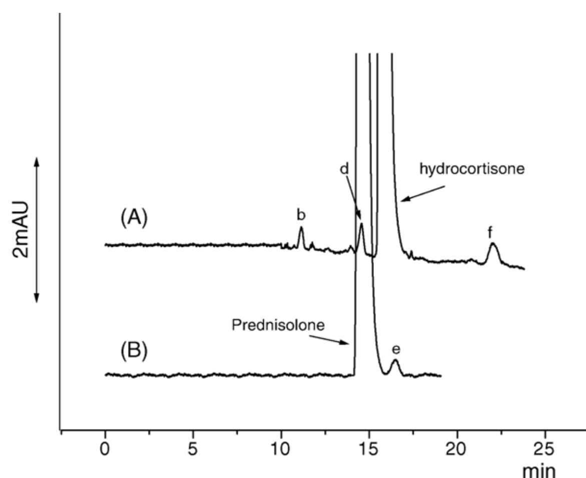

Fig. 7. Electropherograms of mixtures of main drugs and related substances at 0.5% (w/w) level, under MEEKC conditions as in Fig. 6A. (A) Hydrocortisone (main drug), hydrocortisone acetate (b), prednisolone (d) and cortisone (f). (B) Prednisolone (main drug), hydrocortisone (e).

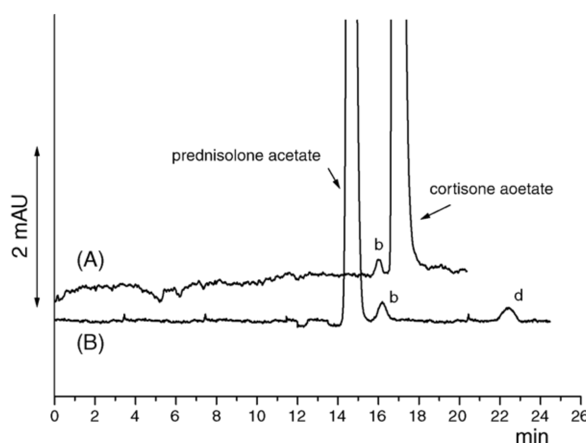

Fig. 8. Electropherograms of mixtures of main drugs and related substances at 0.5% (w/w) level, under MEEKC conditions as in Fig. 6A; the electrophoretic runs were performed at 30 °C. (A) cortisone acetate (main drug), hydrocortisone acetate (b), (B) prednisolone acetate (main drug), hydrocortisone acetate (b) and prednisolone (d).

4. Conclusions

The results obtained suggest that in MEEKC analysis of neutral hydrophobic drugs, such as corticosteroids, the charge density on the surface of the “swollen micelles” is an important parameter affecting separation. In fact, the combined use of SDTC as a favourable alternative surfactant to SDS, with the neutral Brij 76 allowed to easily modulate the separation. However, only by the addition of neutral CDs to this reversed flow MEEKC, adequate separations were achieved. The optimized system provided good preliminary results in the detection of corticosteroids related substances at impurity level.

Acknowledgements

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