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Research Article

# Separation of corticosteroids by microemulsion EKC with diethyl L-tartrate as the oil phase

A novel microemulsion based on a mixture of diethyl L-tartrate (DET) and SDS was developed for the microemulsion EKC (MEEKC) determination of structurally related steroids. The system consisted of 0.5% w/w DET, 1.7% w/w SDS, 1.2% w/w 1-butanol, 89.6% w/w phosphate buffer (40 mM, pH 7.0), and 7% w/w ACN. With an applied voltage of +10 kV, a baseline separation of aldosterone (A), cortisone acetate (CA), dexamethasone (D), hydrocortisone (H), hydrocortisone acetate (HA), prednisolone (P), prednisolone acetate (PA), prednisone (Ps), triamcinolone (T), and triamcinolone acetonide (TA) could be achieved. Under the optimized conditions, the reproducibility of the retention time ($n = 4$) for most of the compounds was less than ±0.8% with the exception of A, Ps, and T. The average number of theoretical plates was 18 800 plates/m. The results were compared with those achieved by the modified micellar EKC (MEKC). MEEKC showed obvious advantages over MEKC for the separation of highly hydrophobic substances. To further evaluate the system, we tested the MEEKC method by analyzing corticosteroids in a spiked urine sample.

**Keywords:**
Corticosteroids / Diethyl L-tartrate / Microemulsion EKC
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## 1 Introduction

Micellar EKC (MEKC) is a very commonly used separation method, due to its simple experimental setup and high separation efficiency. Hundreds of papers related to the method have been published since its introduction by Terabe and co-workers [1, 2]. When microemulsions are employed as the pseudostationary phases (PSPs), the technique is termed microemulsion EKC (MEEKC). MEEKC is similar to MEKC, with the main difference being that the micelles of the microemulsion have tiny oil droplets at their core. Microemulsions have several optimization advantages over other PSPs in terms of selectivity, elution range, composition, and fluidity [3–6]. Hence, it has been proposed that microemulsions have less rigidity than micelles, allowing analytes to penetrate the aggregate more easily and to greater depth.

With the addition of a surfactant and a cosurfactant, the oil droplets are stabilized, and the interfacial tension between the oil and the aqueous phase is reduced [7–12]. Corticosteroids are a class of steroid hormones produced in the adrenal cortex. They are involved in a wide range of physiological processes. Only a few studies concerning MEEKC separation of steroids have been reported [13–18]. Lucangioli *et al.* [16] reported that the microemulsion formed by phosphatidyl choline and isopropyl myristate were apparently better models to estimate the hydrophobicity of the betamethasone series. Pomponio *et al.* [17] reported that sodium taurodeoxycholate (STDC) showed better separation of corticosteroids than the conventional SDS; however, the use of a single anionic surfactant, STDC did not provide the required selectivity. The mix surfactants (Brij 76 and STDC) and CD were found to be essential to obtain adequate resolution. Besides, Gabel-Jensen *et al.* [18] reported major changes in separation selectivity for the charged compounds were observed by modifying the surfactant composition of the microemulsion from pure SDS to mix-surfactants. MEEKC was also accomplished with sodium cholate and cationic surfactants. In contrast, only very small changes in selectivity were indicated for separating the neutral steroids. This might be that they have a large partition to the microemulsion droplets.

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**Abbreviations: A,** aldosterone; **CA,** cortisone acetate; **D,** dexamethasone; **DET,** diethyl L-tartrate; **H,** hydrocortisone; **HA,** hydrocortisone acetate; **MEEKC,** microemulsion EKC; **P,** prednisolone; **PA,** prednisolone acetate; **Ps,** prednisone; **PSP,** pseudostationary phase; **STDC,** sodium taurodeoxycholate; **T,** triamcinolone; **TA,** triamcinolone acetonide

A further increase in separation may be achieved by using a higher concentration of SDS, which leads to additional hydrophobic interaction with the analytes. But an excessive SDS content leads to high operating currents and is therefore avoided. Wu [19] has found good resolution for the mixture of cortisone acetate (CA), hydrocortisone (H), hydrocortisone acetate (HA), prednisolone (P), prednisolone acetate (PA), and prednisone (Ps) in a microemulsion with 0.8% w/w *n*-octane, 3.6% w/w SDS, 6.6% w/w 1-butanol, and 89% w/w phosphate buffer (40 mM, pH 8.0). But long time was needed for the separation. The separation did not improve even with the addition of organic modifier.

Di-*n*-butyl-L-tartrate [20, 21] and ethyl acetate [22–24] have been employed to reduce the interfacial tension in MEEKC separations. In this work we reduced the SDS content by exchanging the oil with diethyl L-tartrate (DET), which might compete less with the analytes. Results from these PSPs, covering all possible variation of the parameters that affect the separations were evaluated. A comparative study in modified MEKC and MEEKC system was also carried out.

## 2 Materials and methods

### 2.1 Chemicals

All chemicals were of analytical reagent grade from Merck (Darmstadt, Germany), unless otherwise stated. Purified water (18 MΩ-cm) from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare all solutions. DET, sodium dihydrogen phosphate, and aldosterone (A) (Acros, Geel, Belgium); 1-butanol (Jassen, Beerse, Belgium); disodium hydrogen phosphate and trisodium phosphate (Merck); benzyl alcohol and methanol (Mallinckrodt, Saint Louis, MO, USA); CA, dexamethasone (D), H, HA, P, PA, Ps, triamcinolone (T), and triamcinolone acetonide (TA) (Sigma, Saint Louis, MO, USA); and phosphoric acid, ACN, and SDS (Wako, Osaka, Japan) were purchased from the indicated sources.

All solvents and solutions for MEEKC analysis were filtered through a 0.45 μm PTFE (Millipore, Billerica, MA, USA) or cellulose acetate (Whatman, Middlesex, UK) membrane.

### 2.2 Instrumentation

All experiments were carried out in a laboratory-built unit consisting of a UV–Vis detector (model UV-2075, Jasco, Japan) and a ±30 kV high-voltage power supply (Gamma High Voltage Research, Ormond Beach, FL, USA). A capillary tube flow cell was used to increase the detection sensitivity (Fig. 1). It was made by removing 10 mm of the polyimide coating and then bending the capillary at two 90° angles. Electropherograms were recorded and processed with a SISC-LAB (32) Ver. 2.01 (Taipei, Taiwan) running on the Windows XP operating system. Separations were carried out in fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm id and an effective length of 30 cm (total length, 53 cm).

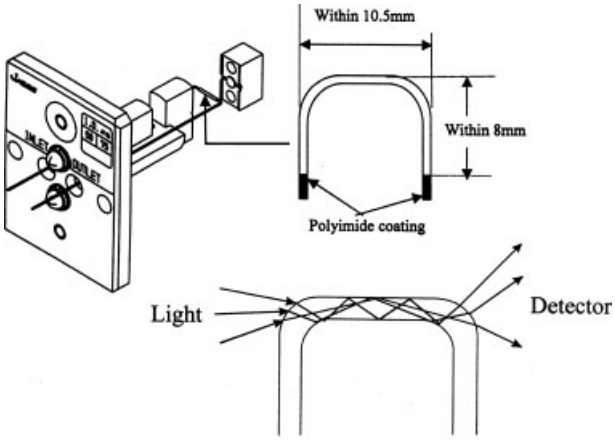


**Figure 1.** Capillary tube detection window.

### 2.3 Sample preparation

Stock solutions of 2 mg/mL for each test compound were prepared in methanol. They were then diluted appropriately with microemulsion and stirred ultrasonically for 5 min before injection.

### 2.4 Microemulsion preparation

Microemulsions were prepared by weighing the desired quantities of surfactant and buffer. The cosurfactant and oil were added next, followed by sonication until the solution became transparent, with no visible droplets on the surface.

### 2.5 MEEKC conditions

Sample introduction was performed hydrostatically (9 cm, 5 s). All analytes were detected by their absorbance at 254 nm.

## 3 Results and discussion

Our first attempts to separate the corticosteroids were carried out using a microemulsion with 0.8% w/w *n*-octane, 3.6% w/w SDS, 6.6% w/w 1-butanol, and 89% w/w phosphate buffer (40 mM, pH 8.0) [19]. Because of the highly hydrophobic and structural similarity of the investigated compounds (Fig. 2), slower migration of the analytes and not sufficiently complete resolution were observed. The phenomenon can be ascribed to the strong nonpolar–nonpolar interaction with the long chain oil phase. To reduce the retention time, oil with lower interfacial tension was used as an alternative.

 

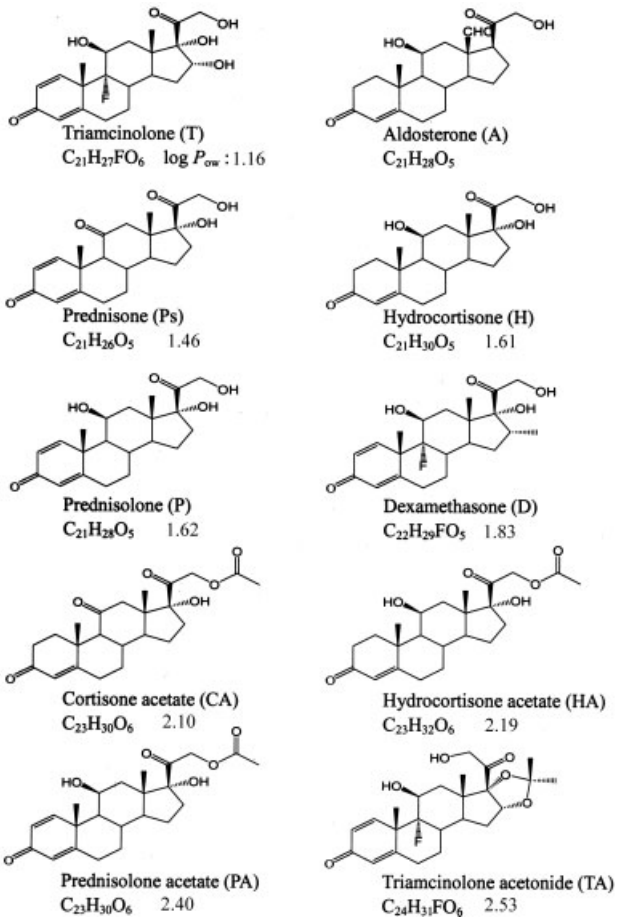


**Figure 2.** Chemical structures of the model compounds (where log $P_{ow}$ is the logarithm of distribution coefficient between 1-octanol and water, data from ref. [26]).

### 3.1 Oil

Di-*n*-butyl-L-tartrate has been used as the oil phase for the separation of ephedrine enantiomer [20, 21]. Ethyl acetate was employed for the separation of Rheum plant extract [22], hematoporphyrin D [23], and nonsteroidal anti-inflammatory drugs [24]. Microemulsions can be produced with a lower SDS content (0.6% w/w), due to the relatively low interfacial tension of these oils. These systems allow the addition of a higher percentage of organic modifiers. Therefore, we tested DET (Fig. 3), an analog of di-*n*-butyl-L-tartrate, as the oil phase in this work. The separation of ten corticosteroids under the conditions of 0.5% w/w DET, 0.6% w/w SDS, 1.2% w/w 1-butanol, and 97.7% w/w phosphate buffer (40 mM, pH 8.0), column length of 53 cm, effective length of 30 cm, and an applied voltage of 10 kV is shown in Fig. 4a. The analysis time was considerably reduced by the comparison with that using octane as oil phase. Although some overlapping occurred between H and P, and among HA, PA and D, as well as between CA and TA, much stable baseline was seen.

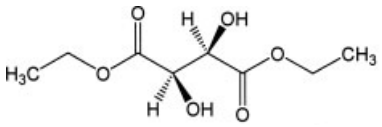

**Figure 3.** Structure of DET.

### 3.2 Concentration of organic modifier

For the purpose of decreasing the total analysis time, ACN has been found to be a better modifier than methanol [19]. We added varying amounts of ACN to a microemulsion system consisting of 0.5% w/w DET, 0.6% w/w SDS, 1.2% w/w 1-butanol, and 77.7–87.7% w/w phosphate buffer (40 mM, pH 8.0). The upper limit was found to be 20% ACN w/w (Fig. 4c). The results indicated that the microemulsion broke down when the ACN content exceeded 20%. Better resolution was obtained at 10% ACN (Fig. 4b).

### 3.3 Concentration of SDS

SDS is the preferred surfactant in most cases. However, attempts to separate some hydrophobic compounds using only SDS have been unsuccessful. More expensive bile salts

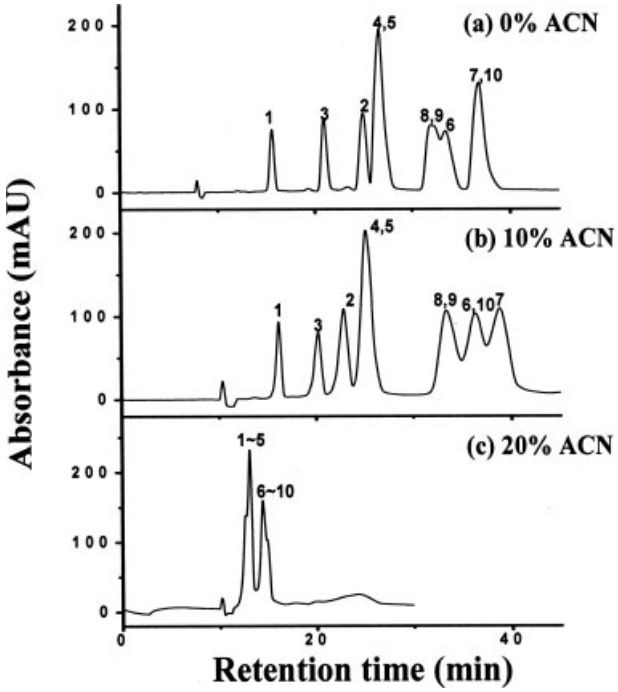


**Figure 4.** Electropherograms of corticosteroids at different concentrations of ACN. Column: fused-silica capillary, 53 cm (30 cm) × 75 μm id; sample injection: hydrostatic (9 cm, 5 s); sample concentration: 58.8 μg/mL for each; applied voltage: +10 kV; detection at 254 nm; mobile phase: 0.5% w/w DET, 0.6% w/w SDS, 1.2% w/w 1-butanol, 77.7-97.7% w/w phosphate buffer (40 mM, pH 8.0), ACN w/w: (a) 0 (b) 10, and (c) 20%. Peak identification: 1, T; 2, A; 3, Ps; 4, H; 5, P; 6, D; 7, CA; 8, HA; 9, PA; 10, TA.

 

are often used [16–18]. In order to investigate the influence of the nature of the surfactant on the migration of the test solute and on the selectivity of the separation, we varied the SDS concentration from 1.0 to 2.0% at pH 8.0, and found 1.7% to be the optimum (Fig. 5). Increasing the SDS concentration even to more than 2.0%, did not improve the resolution of D and TA. This might be due to the excessive high concentration of ACN, resulted in a weak interaction between analyte and microemulsion. We achieved complete resolution of these compounds by decreasing the ACN concentration to 7% (Fig. 6b).

### 3.4 Effect of buffer pH

As pH affects both the EOF velocity and the degree of ionization of solutes, it is a major factor in MEEKC separation of charged compounds. However, corticosteroids are neutral compounds. Generally a pH between 7 and 9 is used to achieve fast, efficient separations [4]. Vomastová *et al.* [13] used more polar long chain alcohol instead of *n*-alkane as the microemulsion-forming agent for the separation of steroids. Although they found the separation was not satisfactory at pHs of 7 and 8, at pH 9 and beyond the separation was successful, we used a phosphate buffer (40 mM) within the range 7.0–9.0 for pH optimization. Increasing the pH resulted in faster elution, especially of the late-eluting analytes (Fig. 6), it severely decreased the resolution of most analytes. We, therefore, chose pH 7.0 for further system optimization trials (Fig. 6a).

### 3.5 Stability of the microemulsion

In order to determine the separation efficiency and the reproducibility of the retention time, replicate injections ($n = 4$) of the sample mixture were carried out (Table 1). The RSD values were quite satisfactory. They were less than 0.8%, except for T, Ps, and A. The average number of theoretical plates was 18 800 plates/m.

### 3.6 Comparison of MEEKC with MEKC

With the conditions as in Fig. 7a, the elution order was T>Ps>A>H>P>HA>PA>D>TA>CA. This did not correspond to the hydrophobicity (log $P_{ow}$ in Fig. 2), which increases in the order T<A<Ps<H<P<D<CA<HA <PA<TA. The difference can be explained by the fact that the oil phase, DET has two hydroxyl groups. The longer retention time of A and D (relative to Ps; HA and PA) might be due to more hydrogen bonding formation with the oil phase. CA having more ketone groups would strongly associate with the PSPs. Therefore, it migrated toward the detector at last.

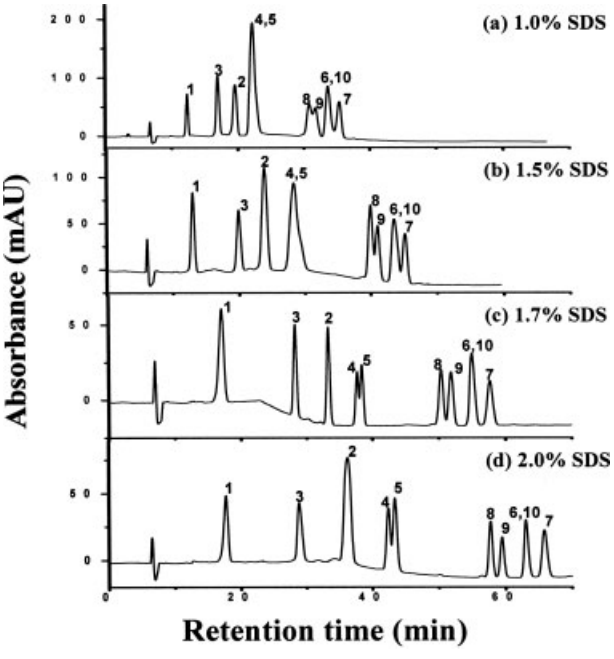


**Figure 5.** Electropherograms of corticosteroids at different concentrations of SDS. Conditions as in Fig. 4 except sample concentration: 30.3 μg/mL for H and P, 58.8 μg/mL for the others; mobile phase: 0.5% w/w DET, 1.2% w/w 1-butanol, 86.3–87.3% w/w phosphate buffer (40 mM, pH 8.0), 10% w/w ACN, SDS w/w: (a) 1.0 (b) 1.5 (c) 1.7, and (d) 2.0%. Peak identification: 1, T; 2, A; 3, Ps; 4, H; 5, P; 6, D; 7, CA; 8, HA; 9, PA; 10, TA.

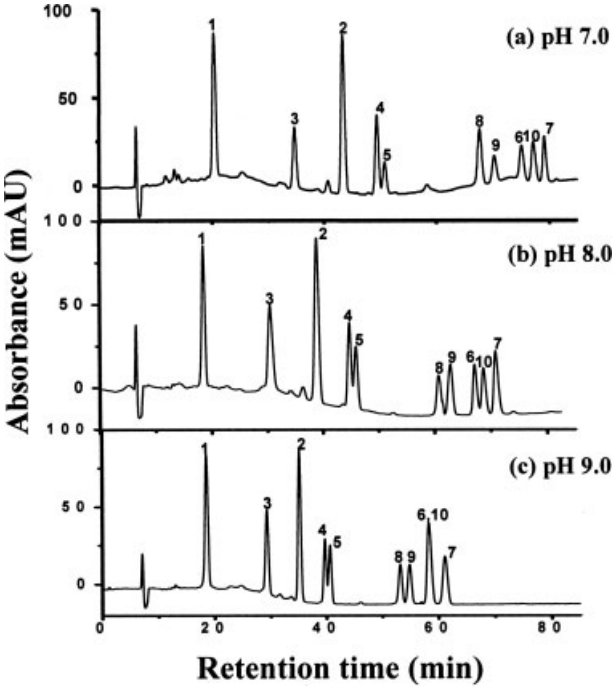


**Figure 6.** Electropherograms of corticosteroids at different pH values. Conditions as in Fig. 5, except that the mobile phase was 0.5% w/w DET, 1.7% w/w SDS, 1.2% w/w 1-butanol, 89.6% w/w phosphate buffer (40 mM), 7% w/w ACN; (a) pH 7.0, (b) pH 8.0, and (c) pH 9.0. Peak identification: 1, T; 2, A; 3, Ps; 4, H; 5, P; 6, D; 7, CA; 8, HA; 9, PA; 10, TA.

 

**Table 1.** Corticosteroid separation efficiencies with the MEEKC method[a)]

| Analytes | pH 7.0 | | pH 8.0 | |
|---|---|---|---|---|
| | $t_R$ (RSD%) (min) | $N$ (plates/m)[b)] | $t_R$ (RSD%) (min) | $N$ (plates/m) |
| MeOH | 6.58 | | 6.50 | |
| T | 20.40 (2.06%) | 2 500 | 18.50 (2.20%) | 1 400 |
| Ps | 34.78 (1.85%) | 2 200 | 30.47 (2.01%) | 700 |
| A | 43.43 (1.21%) | 6 900 | 38.88 (1.30%) | 4 400 |
| H | 49.51 (0.61%) | 18 600 | 44.75 (0.65%) | 20 800 |
| P | 50.89 (0.65%) | 21 600 | 45.93 (0.69%) | 14 600 |
| HA | 67.75 (0.33%) | 20 100 | 60.70 (0.36%) | 10 800 |
| PA | 70.32 (0.14%) | 20 900 | 62.82 (0.15%) | 15 600 |
| D | 75.12 (0.32%) | 26 800 | 67.11 (0.35%) | 24 000 |
| TA | 77.19 (0.73%) | 31 400 | 68.68 (0.80%) | 29 600 |
| CA | 79.14 (0.79%) | 36 900 | 70.83 (0.86%) | 13 900 |

a) Column: fused-silica capillary, 53 cm (30 cm) × 75 μm id; sample injection: hydrostatic (9 cm, 5 s); sample concentration: 30.3 μg/mL for H and P, 58.8 μg/mL for others; applied voltage: +10 kV; detection at 254 nm; mobile phase: 0.5% w/w DET, 1.7% w/w SDS; 1.2% w/w 1-butanol, 89.6% w/w phosphate buffer (40 mM), 7% w/w ACN.

b) Theoretical plate, $N = 5.54\ (t_m/W_{1/2})^2$.

The results were quite different from that of octane and SDS-containing system, where H and P could not be resolved at all, also Ps was eluted after H and P [14].

By careful consideration of all the results shown in Fig. 7, it is interesting to note that the elution order was the same for both MEEKC and the modified MEKC (the microemulsion without the oil phase). But the resolution of H and P, as well as TA and CA became poor in the modified MEKC. The results indicated that the high selectivity of the MEEKC system might be mainly attributed to the proper choice of the oil phase. The larger relative volume of PSP to aqueous phase compared with MEKC results in better separation efficiency [25].

### 3.7 Application

The refined method was applied to the analysis of corticosteroids after they were spiked to a urine sample. Here, a fourfold dilution of the sample in the microemulsion was injected directly, without any pretreatment. The electropherogram is shown in Fig. 8. Among the analytes, the resolution values were still good and suitable for quantitative analysis, with the exception of D, CA, and TA.

## 4 Concluding remarks

The complexity of the microemulsion composition and the MEEKC separation process allow many manipulations to be made in order to achieve a particularly difficult resolution [7].

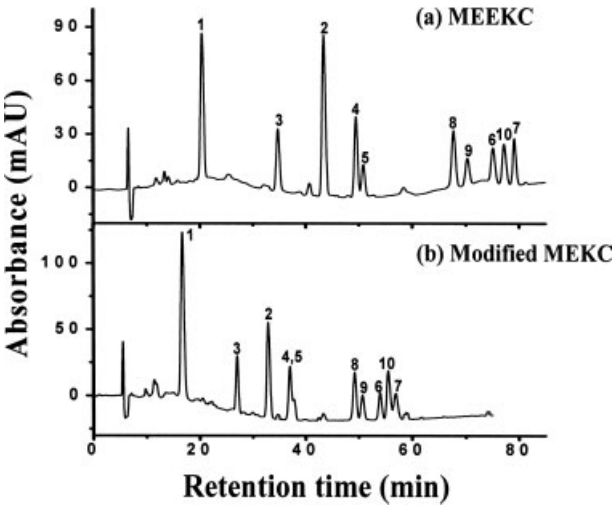


**Figure 7.** Comparison of the separation of corticosteroids by MEEKC and by modified MEKC. Conditions as in Fig. 5, except that the mobile phase was 1.7% w/w SDS, 1.2% w/w 1-butanol, 89.6–90.1% w/w phosphate buffer (40 mM, pH 7.0), 7% w/w ACN; (a) 0.5% w/w DET, MEEKC and (b) 0% w/w DET, modified MEKC. Peak identification: 1, T; 2, A; 3, Ps; 4, H; 5, P; 6, D; 7, CA; 8, HA; 9, PA; 10, TA.

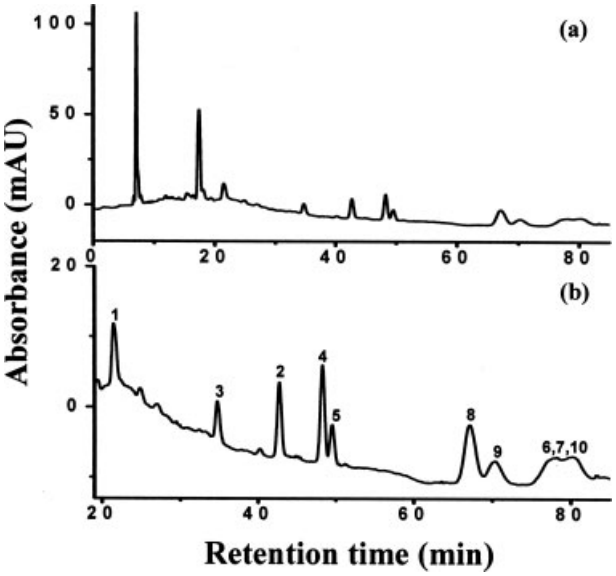


**Figure 8.** Electropherograms of a urine sample spiked with corticosteroids. Conditions as in Fig. 5, but with the following sample concentrations: 4.0 μg/mL for the first five steroids, 10.7 μg/mL for the others, and mobile phase; (a) 0.5% w/w DET, 1.7% w/w SDS, 1.2% w/w 1-butanol, 89.6% w/w phosphate buffer (40 mM, pH 7.0), 7% w/w ACN, (b) enlarged spectra of (a). Peak identification: 1, T; 2, A; 3, Ps; 4, H; 5, P; 6, D; 7, CA; 8, HA; 9, PA; 10, TA.

Here, we found a novel microemulsion based on DET at pH 7.0 to be suitable for the separation of corticosteroids. The separation efficiency was better than that attained with the modified MEKC method (without the oil phase). This

 

might be explained by greater interaction between the PSPs and the analyte in MEEKC. The analytes can penetrate the aggregate of the microemulsion more easily than they can do in the micelle, resulting in sufficient partitioning difference between the analytes in MEEKC. The property made the separation of highly hydrophobic and structurally correlated compounds feasible. We also found that the established methods are effective and promising for the separation of these analytes in a biological fluid.

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