Separation of Steroids by Micellar Electrokinetic Capillary Chromatography with Sodium Cholate

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Abstract

Several steroid hormones that are routinely determined in clinical settings are resolved by micellar electrokinetic capillary chromatography (MECC) in phosphate buffer (pH = 7.4, 15 kV). These steroidal compounds are separated by MECC within a short time under physiological conditions via the use of bile salt surfactant (0.04M sodium cholate) in conjunction with an appropriate organic modifier (20% methanol). The elution patterns of seven steroid hormones are determined within 9 min with a voltage of 30 kV. The effect of varying the percentage of added methanol modifier, the applied voltage, and the temperature on the migration behavior of the steroids is examined.

Introduction

Steroid hormones, which constitute one subset of the body's chemical communication system, are derivatives of cholesterol. Their structures are very similar (Figure 1). In the clinical diagnosis, the concentration of steroid hormones in the serum is often an indication of endocrine abnormalities that cause diseases such as renovascular hypertension, congenital adrenal hyperplasia (CAH), and Cushing's syndrome (1). Radioimmunoassay kits are widely used in diagnostic examinations. Although these kits are commercially available, they are too expensive, and the method is too time-consuming. To remedy this, gas chromatography (GC) and high-performance liquid chromatography (HPLC) were proposed, which give a total profile of multiple steroids and are mostly used for endocrine research (2–4). Unfortunately, these methods are still rather time-consuming (30 min to over an hour) and require optimization of the analytical conditions for each individual researcher. Moreover, the diagnostic results of free hormones may vary from one laboratory to another due to interference caused by impurities and running buffers of extraction processes (5). To avoid the aforementioned shortcomings, this study reports a new approach that uses capillary electrophoresis (CE) to separate the steroids.

CE can be utilized to analyze a wide variety of charged and uncharged species. Sizes range from those of small analytes such as metal ions to large molecules such as amino acids, proteins, DNA, and RNA (6–12). The results have confirmed its high proficiency and accuracy. Capillary zone electrophoresis (CZE) separates ionic compounds of different charge-to-mass ratios. Nonionic analytes are not resolved by CZE.

In 1984, Terabe (13,14) added surfactants to modify the CZE conditions. In this method, a micelle pseudophase was used for separating the substances in CE; the method is therefore referred to as micellar electrokinetic capillary chromatography (MECC). MECC separations are based on a differential partitioning of analytes between the solvent phase and micellar phase. Sodium dodecyl sulfate (SDS), as an electrolyte additive in MECC, has proven very useful for separation of water-soluble analytes (e.g., ascorbic acid [15]) and nonionic molecules (16,17). Some hydrophobic compounds were separated with the addition of urea and acetonitrile in an SDS solution (18,19). But SDS micelles have a limited elution range. Shi and Fritz (16,17) reported the separation of hydrophobic compounds such as PAHs and nonionic aromatic compounds (e.g., acetophone, azulene, etc.) using quaternary ammonium salt, percentage variation of acetonitrile, sodium dioctyl sulfosuccinate, and pH. The effect of cation buffer was reported by Chen and Pietrzyk for separation of sulfonate and sulfate surfactants by CE (20). Cole et al. (21) adopted bile salts in the mobile phase instead of SDS and optimized the resolution of binaphthyl enantiomer separation successfully.

Experimental

Instrumentation

A Beckman 5500 P/ACE electrophoresis system equipped with Gold software for data collection (Beckman, Fullerton, CA) was employed for CE. The P/ACE system included a temperature-controlled cartridge enclosing a capillary column,

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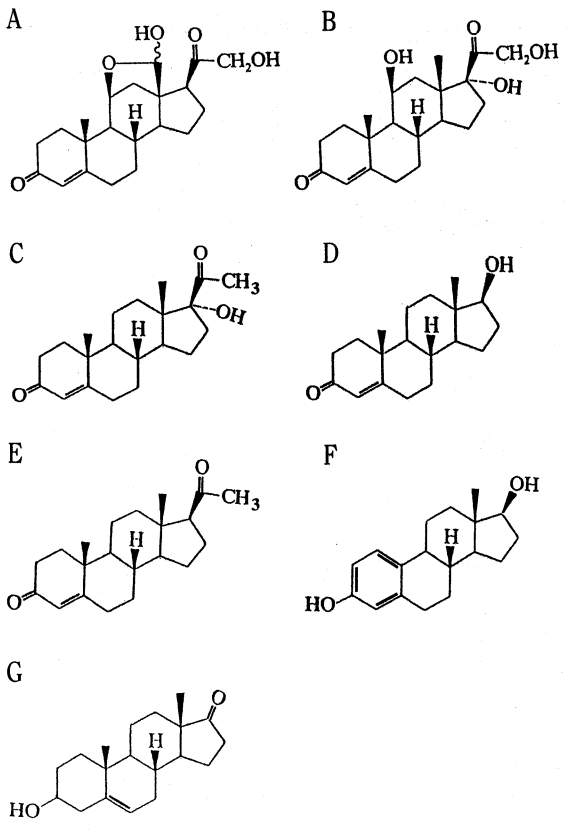

Figure 1. Structures of seven steroid hormones. (A) aldosterone, (B) cortisol, (C) 17-α-OH-progesterone, (D) testosterone, (E) progesterone, (F) estradiol, (G) dehydroepiandrosterone.

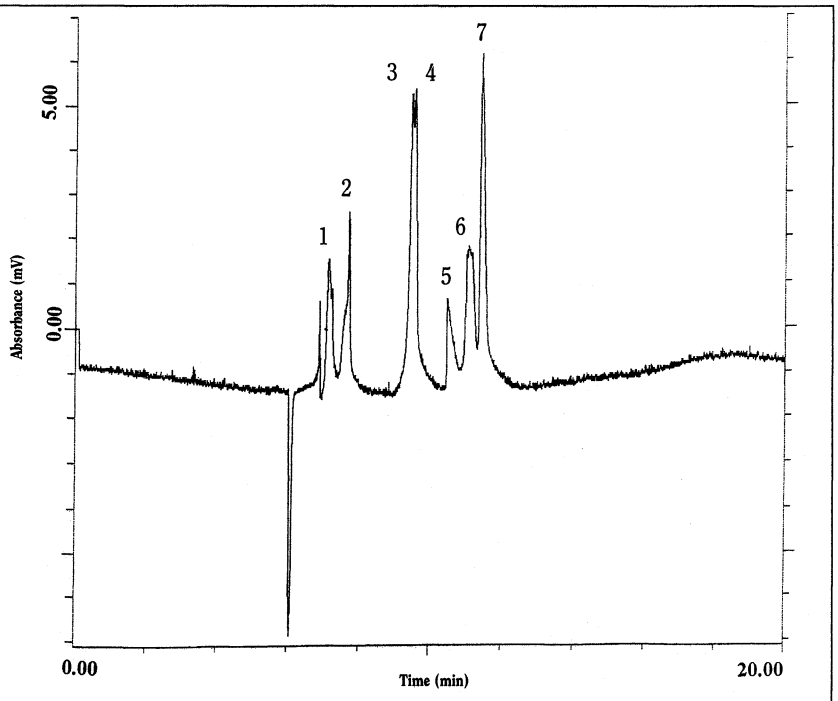

Figure 2. Electropherogram of seven steroid hormones in 0.038M phosphate buffer solution with 0.04M sodium cholate. No methanol added. pH = 7.4; temperature, 25°C; voltage, 15 kV. Peaks: 1, aldosterone; 2, cortisol; 3, 17-α-OH-progesterone; 4, testosterone; 5, dehydroepiandrosterone; 6, progesterone; 7, estradiol.

an autosampler, a wavelength-selectable detector, and an electric interface. Spectra were collected with the use of the 168 diode-array detector using the "scan graphic" option. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) used for CE had 50-µm inner diameters and 350-µm outer diameters. The capillaries were 50-cm long between the injection end and the detection window. The whole length of the capillary between the inlet and outlet was 57-cm long. The P/ACE instrument was controlled automatically via an IBM-compatible personal computer with system Gold software. Data were collected with the P/ACE software system. This package provided automated instrument control and sophisticated data analysis capability.

Reagents

Aldosterone, cortisol, 17-α-hydroxyprogesterone, testosterone, progesterone, estradiol, and dehydroepiandrosterone sulfate were obtained from Sigma (St. Louis, MO). The testosterone was interconvertible to androsterodione and vice versa, and it was obtained from Fluka Chemical (Buchs, Switzerland). Sodium monophosphate, sodium diphosphate, sodium hydroxide, sodium cholate, sodium tetraborate, SDS, and methanol were from Sigma (St. Louis, MO). All chemicals were of analytical grade or better and were used as received without further purification. They were dissolved in doubly distilled water obtained from a Millipore (Bedford, MA) water purification system with at least 18.2 MΩ of water resistance.

Procedure

Stock solutions of steroid hormones were prepared independently by dissolving different analytes in methanol in the concentration range of 2.5–10 mg/mL. Sample solutions of steroid hormones were obtained by diluting the stock solutions in running buffers to reach the concentration of 0.25–2.0 mg/mL, which gave an adequate peak height. Running buffers were prepared by dissolving sodium monophosphate and sodium diphosphate to give a pH of 7.4. Varied concentrations of methanol and sodium cholate were added in the phosphate buffer solutions for running CE. Initially, the new capillary was rinsed with 1N HCl for 5 min and then with a regenerated solution (0.1N NaOH) for 10 min before it was rinsed with deionized water for 5 min. Samples were introduced to the column by pressure injection for 5 s, corresponding to a 3.1 nL volume. After each run, the capillaries were rinsed with the 0.1N NaOH solution for 10 min followed by deionized water for 5 min. After the buffer was changed, separation voltage was applied for 10 min to condition the column with the new buffer. All the surfactant solutions were filtered through 0.45-µm membrane filters.

Results and Discussion

Steroids are very lipophilic, not soluble in water, and electrically neutral in the range of pH 7–9. They are soluble in methanol and other organic solvents. Using CZE did not resolve them in the pH 7–9 (phosphate–borate buffer) range. The migration times of the electroosmotic flow (EOF), steroids, and methanol were about the same. In this pH range, the peaks were very broad and tailed. This may indicate adsorption of the analytes on the capillary wall.

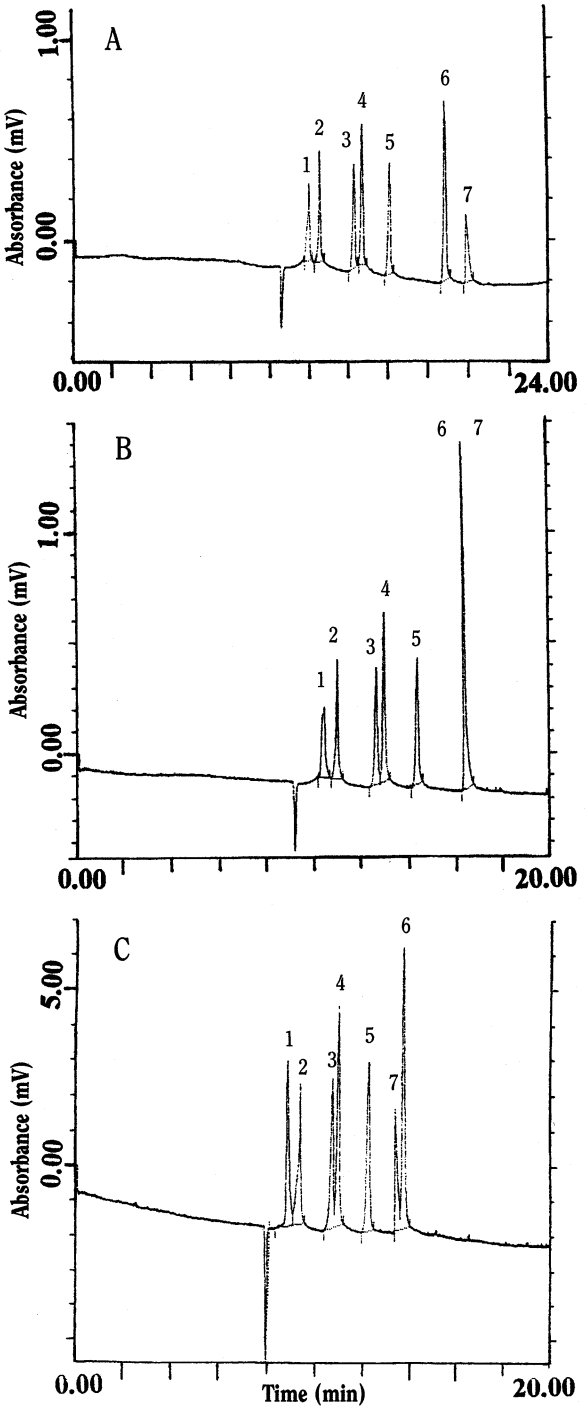

Figure 3. Electropherogram of steroid hormones in 0.038M phosphate buffer solution with 0.04M sodium cholate and (A) 20%, (B) 15%, and (C) 10% (v/v) methanol. pH = 7.4; temperature, 25°C; voltage, 15 kV. Peaks: 1, aldosterone; 2, cortisol; 3, 17-α-OH-progesterone; 4, testosterone; 5, progesterone; 6, estradiol; 7, dehydroepiandrosterone.

A surfactant is an amphiphilic molecule, one end of which is a polar or ionic group and the other end of which is hydrophobic. In an aqueous media, micelles form. The solubility of insoluble nonpolar compounds in the aqueous solution is enhanced due to the interaction between the micelle hydrophobic core and the sparingly water-soluble molecule. The interaction mechanisms between micelles and solutes can be surface adsorption, partitioning, and comicellization. MECC separation relies on the differences in the extent of interaction between micelles and solutes but not micellar solubilization. The separation mechanisms of MECC involve hydrophobic interactions, electrokinetic migration, and differences in solute partitioning to the micelle.

Separation of these highly hydrophobic steroid hormones by MECC with addition of SDS in the phosphate–borate buffer solutions did not achieve separation in the buffer range of pH 7–9. Their migration time was almost the same with 0.02M–0.1M SDS in the solution. In CE, the resolution, separation proficiency, selectivity, and elution time of the analytes can be simply optimized by the capacity factor, k' (e.g., assorted surfactants, surfactant concentrations, organic modifiers, temperature, voltages, etc.). The resolution of MECC changes. This can be caused by the influence of the migration factor, and to some extent, the elution window width as the micelle migration changes. Micelle concentrations also have an effect on the resolution in MECC. Selectivity is closely related to different partitioning and binding sites between micelles and analytes, but it is not affected by micelle concentration.

MECC with bile salt

Figure 2 shows the MECC electropherogram of the mixture of seven steroid hormones examined routinely in the hospital with the addition of 0.04M sodium cholate in the 0.038M phosphate buffer at a pH of 7.4. Separation of these steroids was not totally successful under the electrophoretic mobility of the experimental conditions. Six peaks were detected in the electropherogram. Two of them (Figure 2, peaks 3 and 4) had almost the same mobility and could not be distinguished.

Table I. Migration Time of Steroid Hormones at Different Percentages of Methanol in 0.038M Phosphate Buffer with 0.04M Sodium Cholate at 25°C and 15 kV

	Migration time (min)			
	0% methanol	10% methanol	15% methanol	20% methanol
Aldosterone	7.17	8.84	10.44	11.97
Cortisol	7.73	9.37	10.98	12.57
17-α-OH-Progesterone	9.53	10.76	12.65	14.34
Testosterone	9.61	11.02	12.98	14.75
Progesterone	11.13	12.27	14.36	16.14
Estradiol	11.48	13.76	16.33	18.91
Dehydroepiandrosterone	10.54	13.42	16.33	19.09

Variations of the sodium cholate from 0.02 to 0.1M were attempted in order to resolve the mixture of analytes in CE but were not successful.

MECC with bile salts and organic modifier

The migration factor can be adjusted through the addition of organic modifiers in the micellar solution to improve the partition selectivity. This may expand the elution window in the electropherogram. Also, the partition coefficients of analytes into micelles, EOF velocity, and micellar migration time may be changed by the additive. For compounds with identical electrophoretic mobility, the addition of some organic media can lead to selectivity enhancement. In MECC, adding organic modifiers to the micellar solution to improve the resolution of highly hydrophobic compounds was first reported by Balchunas et al. (22) and later by Sepaniak et al. (23) and Gorse et al. (24).

The use of organic modifiers in the operating solutions has been reported to be effective for the separation of some organic compounds by reducing the capacity factor (21,25). However, the separation efficiency did not appear to be improved much by the addition of organic modifiers in the SDS micelle solution, although it did increase the migration time due to the decreased EOF.

A typical electropherogram of seven hormones in MECC after the addition of 20% methanol in bile salts (0.04M sodium cholate) as the running buffer solution (0.038M phosphate, pH 7.4) is shown in Figure 3A. All seven hormonal solutes eluted under the described conditions at 25°C and 15 kV within 20 min. The addition of methanol in the running buffer solutions caused the extension of the solutes to extend the elution time by decreasing the EOF.

The driving force of the EOF arose from the capillary wall. Thus, dynamic modification of the wall through the addition of the organic chemicals could change the EOF. Buffer conductivity and EOF commonly decrease upon the addition of organic modifiers, and this results in the increase of on-capillary time. Thermal diffusion decreases as the concentration of the organic additives increases. Such effects were observed in the electropherogram of Figure 3A, in which the seven steroid hormones were resolved in a different elution time. Also, the addition of methanol improved the solubility of some analytes that did not dissolve well in aqueous solutions. Different steroids exhibited different behavior with the addition of methanol. The migration time changed because all the analytes were sensitive to the presence of methanol. Dehydroepiandrosterone exhibited a larger change in elution time than the other steroids. Its migration time increased from 10.54 min to 13.42 min when the concentration of methanol was changed from 0 to 10% (see Table I).

Figure 3B shows the electropherogram of steroids after 15% of methanol was added in the running 0.038M phosphate buffer

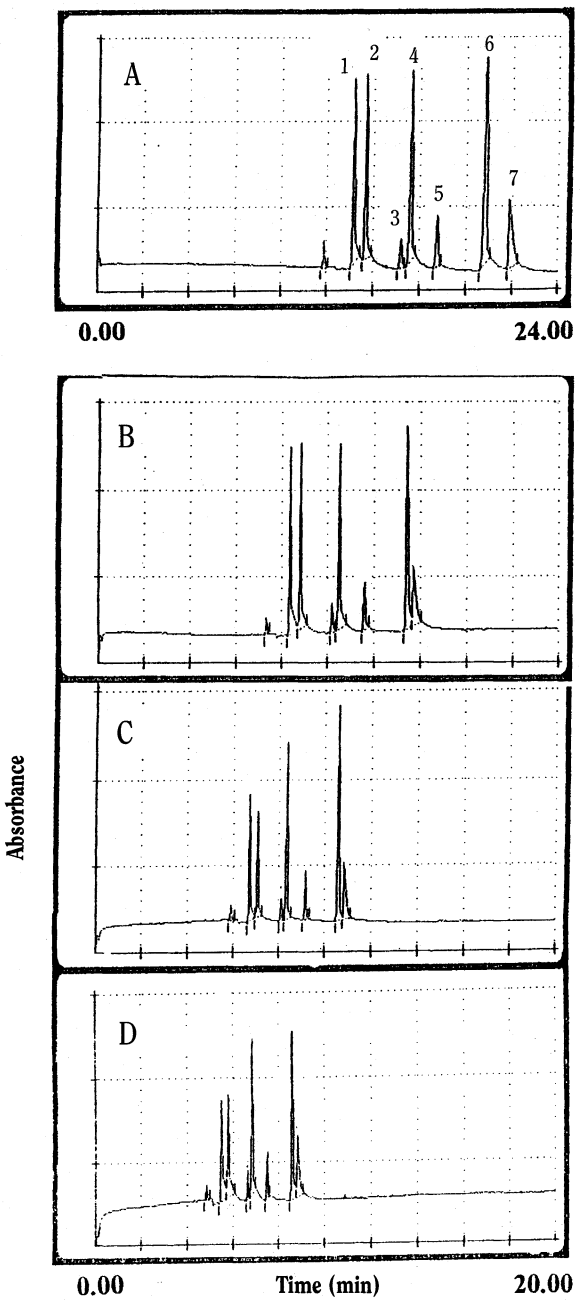

Figure 4. Electropherogram of hormones in 0.038M phosphate buffer solution with 20% methanol in 0.04M sodium cholate. (A) 10 kV, (B) 20 kV, (C) 25 kV, (D) 30 kV. pH = 7.4, temperature, 25°C. Peaks: 1, aldosterone; 2, cortisol; 3, 17-α-OH-progesterone; 4, testosterone; 5, progesterone; 6, estradiol; 7, dehydroepiandrosterone.

Table II. Migration Time of Steroid Hormones at Different Applied Voltages with 20% Methanol in 0.038M Phosphate Buffer and 0.04M Sodium Cholate at 25°C

	Migration time (min)			
	10 kV	20 kV	25 kV	30 kV
Aldosterone	13.45	8.36	6.69	5.47
Cortisol	14.07	8.83	7.05	5.76
17-α-OH-Progesterone	15.93	10.20	8.11	6.61
Testosterone	16.43	10.52	8.36	6.81
Progesterone	17.81	11.60	9.19	7.48
Estradiol	20.31	13.41	10.59	8.60
Dehydroepiandrosterone	21.54	13.68	10.83	8.82

solutions with 0.04M sodium cholate. The last peak in Figure 3B (peak 6) corresponds to estradiol and dehydroepiandrosterone. This pair of hormones was not resolved. However, estradiol and dehydroepiandrosterone were resolved as two peaks in Figures 3A and 3C. Methanol was added at 20% in the running buffer solution in Figure 3A, and 10% of methanol was added to the running buffer solution in Figure 3C. Peaks 1 and 2, 3 and 4, and 6 and 7 in Figure 3C are partially overlapped.

Note that the migration pattern of dehydroepiandrosterone is before that of estradiol in the electropherogram of Figure 3C (10% methanol), and the migration pattern of dehydroepiandrosterone is after estradiol in the electropherogram of Figure 3A (20% methanol). This phenomenon may be due to the interaction among the micellar hydrophobic core, methanol, and steroids themselves. The same electrophoretic mobility may result from the total migration factors (e.g., EOF velocity, partition coefficients, micellar migration time, etc.). The reason why such behavior occurred is unclear and requires further study.

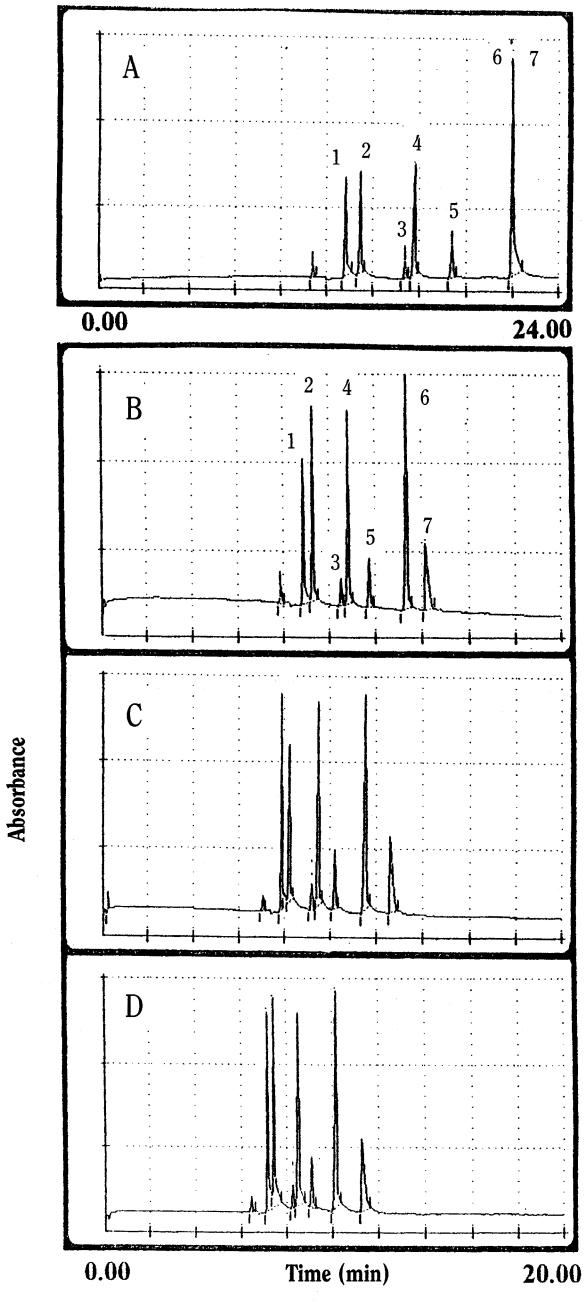

Figure 5. Electropherogram of hormones in 0.038M phosphate buffer solution with 20% methanol in 0.04M sodium cholate. (A) 20°C, (B) 35°C, (C) 40°C, (D) 45°C. pH = 7.4, voltage, 15 kV. Peaks: 1, aldosterone; 2, cortisol; 3, 17-α-OH-progesterone; 4, testosterone; 5, progesterone; 6, estradiol; 7, dehydroepiandrosterone.

Variation of voltage

Variation of voltage and changing the electrical field can have several effects (e.g., variation in the migration time of samples, EOF, analysis time, resolution, peak sharpness, and joule heating). The joule heating that results from an increase in voltage might lead to changes in EOF, ion mobility, analyte diffusion, and band broadening. Figure 4 shows the electropherogram of the steroids running in 0.038M phosphate buffer solutions with 0.04M sodium cholate and 20% methanol at different applied voltages. Seven peaks are shown in Figure 4. The migration time decreased when the applied voltage was increased. For example, the migration time of dehydroepiandrosterone decreased from 21.54 to 8.82 min (Table II) when the applied voltage was increased from 10 to 30 kV (Figure 4). The elution time in this study was shortened due to the increased velocity of the analytes and EOF. The sequence of the elution order did not change with different applied voltages. The joule heating effect (e.g., peak broadening) was not observed.

Temperature effect

Temperature increases can lead to a decreased viscosity of the running buffer and an increase in the electrophoretic mobility of analytes and EOF. Conformation of analytes may change due to the variation of the temperature. Some analytes may not be stable at higher temperatures. Also, the volume of some types of injected samples may not be constant because the buffer viscosity is varied at different temperatures, especially when the injection is by the hydrostatic method (e.g., pressure). Figure 5 shows the electropherograms of the steroids running in 0.038M phosphate buffer solutions with 0.04M sodium cholate and 20% methanol at different applied temperatures. Only six peaks were detected in the electro-

Table III. Migration Time of Steroid Hormones at Different Temperatures of 20% Methanol in 0.038M Phosphate Buffer with 0.04M Sodium Cholate at 15 kV

	Migration time (min)			
	20°C	35°C	40°C	45°C
Aldosterone	12.96	8.87	7.88	7.14
Cortisol	13.73	9.28	8.21	7.42
17-α-OH-Progesterone	16.05	10.50	9.20	8.25
Testosterone	16.54	10.84	9.496	8.50
Progesterone	18.45	11.74	10.20	9.08
Estradiol	21.50	13.36	11.54	10.15
Dehydroepiandrosterone	21.50	14.15	12.59	11.26

pherogram in Figure 5A at a temperature of 20°C. Seven peaks are shown in the electropherograms in Figure 3A and Figures 5B–5D at 20–45°C. The apparent effects of band broadening, sample denaturation, sample content increase for analysis, sample stability problems, and confirmation change were not resolved in the electropherograms of Figure 5. However, increased EOF and electrophoretic mobility could result in a shorter analysis time. The migration times of seven steroid hormones in different applied temperatures with 20% methanol in the running buffer solutions can be seen in Table III.

Estradiol and dehydroepiandrosterone were not resolved in the electropherograms at a lower temperature shown in Figure 5A. The area of the last peak in Figure 5A is approximately equal to the area of peak 6 (estradiol) and peak 7 (dehydroepiandrosterone) in Figure 5B. At 20°C, estradiol migrated at the same velocity as dehydroepiandrosterone. When the temperature was increased, the speed of estradiol was faster than that of dehydroepiandrosterone in the running conditions. The elution time of other steroids shifted regularly as temperature increased with increasing migration speed. Further work is needed to clarify the effects of temperature.

Conclusion

In conclusion, it was found that seven steroids could be successfully separated by MECC using sodium cholate and the addition of methanol. This MECC approach can potentially be applied in clinical, pharmaceutical, or biological settings.

Acknowledgments

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