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Short communication

Separation of estrogens by micellar electrokinetic chromatography

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Abstract

Capillary electrophoresis of the sex hormone estrogens using different buffer components was investigated. Free zone electrophoresis with 10 m*M* phosphate buffer (pH 11.5) or 10 m*M* phosphate buffer with 10–20% methanol was not effective in separating the ten estrogens used in this study. However, nine estrogens were resolved by micellar electrokinetic chromatography using a 10 m*M* borate buffer (pH 9.2) containing 100 m*M* sodium cholate. In addition, some estrogens were partially separated using sodium dodecyl sulfate (SDS) micellar buffers; however, the addition of modifiers such as organic solvents or cyclodextrins improved resolutions significantly. Using a 10 m*M* phosphate buffer (pH 7.0) containing 50 m*M* SDS and 20% methanol, or a 10 m*M* borate buffer (pH 9.2) containing 50 m*M* SDS and 20 m*M* γ-cyclodextrin, all ten of the tested estrogens were separated. However, the cyclodextrin-modified buffer allowed faster separation.

1. Introduction

Estrogens are steroidal sex hormones formed from the precursors androstenedione and testosterone [1]. The most potent naturally occurring estrogen is 17β-estradiol, which is interconvertable to the less potent compound, estrone. Both of these estrogens can also be metabolized to estriol, which also has limited estrogenic activity. All of these transformations take place mainly in the liver. Estrogen determinations are widely used to monitor pregnancy [2], osteoporosis [3], breast cancer [4] and uterine corpus cancer [5]. Radioimmunoassay [6] or enzyme immunoassay [7] have been used for the sensitive detection of estrogens. Also, chromatographic methods allow simultaneous determinations of various estrogens. High-performance liquid chromatography (HPLC) with UV absorption [8–11], fluorescence [12] or electrochemical detection [13,14], gas chromatography or gas chromatography–mass spectrometry [15–17] and supercritical fluid chromatography have been used for the analyses of estrogens [18]. Estrogens prelabeled with a 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole have also been separated by HPLC followed by fluorescence detection [19]. In addition, post-column chemiluminescence detection of estrogen derivatized with dansyl chloride was also demonstrated [20].

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Due to its high resolution, rapid separation, low mass detectability, and easy operation, capillary electrophoresis (CE) has recently emerged as a very attractive separation tool complementary to HPLC [21]. Capillary zone electrophoresis (CZE) has been used exclusively for the separations of charged compounds. Another mode of CE, micellar electrokinetic chromatography (MEKC), is widely used for the separation of non-polar molecules [22]. This technique uses buffers containing organic micelles to separate analytes based on their differential partitions between the aqueous and the micellar phases.

Separations of steroidal compounds using MEKC have been demonstrated. For example, MEKC using sodium dodecyl sulfate (SDS) micelles was applied to the separation of the insect and plant hormones, ecdysteroids [23]. In addition, MEKC or MEKC modified with urea or cyclodextrins (CDs) were applied to the separations of corticosteroids [24–27]. MEKC of corticosteroids using mixed micelles was also shown [28]. Recently, separation of three estrogens by CZE using aqueous or aqueous–methanolic buffers was reported [29]. In this study, separations of estrogens under different MEKC conditions were investigated.

2. Experimental

2.1. Chemicals

Estrogen standards were obtained from Steraloids (Wilton, NH, USA). Stock estrogen solutions were prepared in methanol and stored at −20°C when not in use. CDs were obtained from Advanced Separation Technologies (Whippany, NJ, USA). SDS, sodium cholate, sodium deoxycholate and other reagent-grade chemicals were obtained from Sigma (St. Louis, MO, USA) or Fisher (Fairlawn, NJ, USA). All buffers were filtered with a 0.2-μm membrane filter before use.

2.2. Apparatus

A Beckman P/ACE 2050 CE system was used. Separations were performed at 20°C and +17 kV with 47 cm × 50 μm fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). Injection samples were prepared by diluting the estrogen stock solutions in either 50 m*M* of SDS, sodium cholate or sodium deoxycholate. Samples were introduced into a capillary by applying pressure (0.5 p.s.i.; 1 p.s.i. = 6894.76 Pa) for 2 s. The capillaries were flushed with the electrophoretic buffers for 2 min between runs. Peaks were detected by UV absorption at 200 nm.

3. Results and discussion

The most potent estrogen in humans is 17β-estradiol, followed by estrone and estriol. Each of these molecules contain a 17-carbon nucleus (steroid) with a methyl group at C-13, and an aromatic ring with a hydroxyl group at C-3 (Fig. 1). The derivatives are designated by the presence of a ketone (estrone) or hydroxyl (estradiol) group at C-17 and frequently at C-16 (16-keto-17β-estradiol, estriol). Estrogens are negatively charged at alkaline solution due to the ionization of the phenolic hydroxyl group. CZE has been used to separate 17β-estradiol, estrone and estriol [29]; but it was not adequate in resolving the ten estrogens used in our study (Fig. 2a) because these estrogens have similar electrophoretic mobilities. Although the addition of organic solvents to running buffers is a common method for enhancing separations by lowering electroosmotic flow and increasing solvation of solutes [28–30], separation of most estrogens was still unsuccessful using buffers containing 10–20% methanol (Fig. 2b and c).

MEKC of estrogens using bile salts as micelles was investigated. Using a 10 m*M* borate buffer (pH 9.2) containing 100 m*M* deoxycholate, partial separation of the estrogens was possible (Fig. 3a), while nine estrogens were separated using 100 m*M* sodium cholate (Fig. 3b). 4-Hydroxyestrone and 2-hydroxyestradiol were not resolved using the indicated running conditions. MEKC of estrogens using SDS micelles was also investigated. In contrast to the bile salts, SDS micelles strongly retain the estrogens, resulting in poor separation (Fig. 4a). Organic modifiers are often used in MEKC to decrease the af-

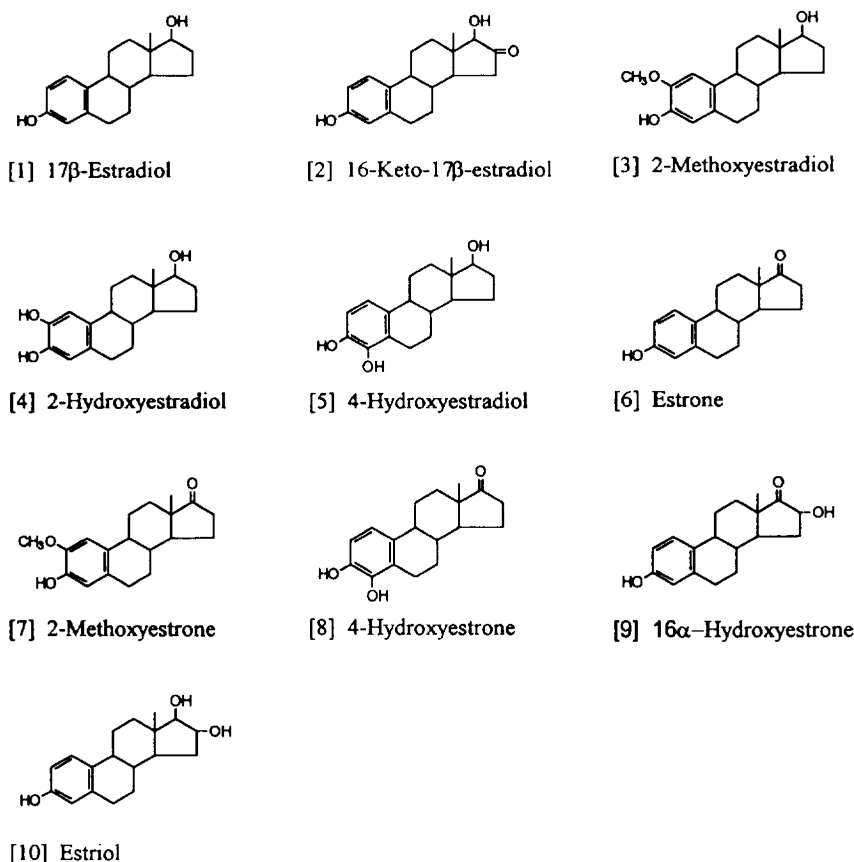

Fig. 1. Chemical structures of ten estrogens.

finities of hydrophobic solutes for the micellar phase. In addition, organic solvents reduce electroosmotic flow and subsequently expand the migration window [31–34]. As a result, resolution of highly hydrophobic compounds in MEKC is enhanced. Addition of 15% acetonitrile to the SDS buffer allows for the separations of all estrogens, except 4-hydroxyestrone and 4-hydroxyestradiol (Fig. 4b), while all ten estrogens were separated with 20% methanol (Fig. 4c).

SDS micellar buffers modified with CDs have been used to separate the highly hydrophobic compounds, corticosteroids [25–27], which share the steroidal nucleus of estrogens. CD reduces the capacity factors for the micellar phase by forming inclusion complexes with corticosteroids. The separations are based on the differential partitions of the solutes among the aqueous phase, CD, and SDS micelles. Compared to corticosteroids, estrogens are more hydrophilic because of the presence of phenolic hydroxyl groups. Nevertheless, CD also plays an important role in the MEKC of estrogens, as shown in Fig. 5a–c. Most of the ten estrogens were baseline separated with a 10 mM borate buffer (pH 9.2) containing 50 mM SDS and 20 mM β-CD, while the addition of 20 mM γ-CD in the SDS buffer allowed for the complete separation of the ten estrogens. The pair consisting of 2-methoxyestradiol and 2-methoxyestrone, which were partially resolved with the β-CD, were baseline separated with the γ-CD-modified buffer. This is because the larger size of the methoxy derivatives require a CD with a larger cavity (i.e., γ-CD) for more effective interaction. The migration orders of most estrogens were similar in either CD-modified buffers, except that the peaks of 16α-hydroxyestrone and 16-keto-17β-estradiol were reversed. MEKC modified with α-CD was also attempted, but

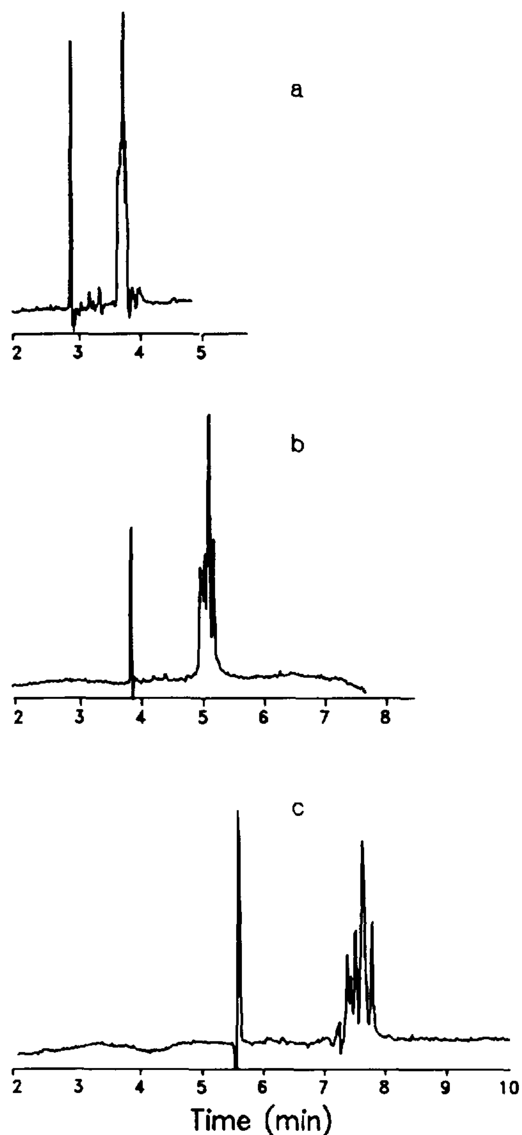

Fig. 2. CZE of ten estrogens. Buffers: 10 m*M* sodium phosphate (pH 11.5) containing (a) 0%, (b) 10% and (c) 20% of methanol; capillary: 47 cm × 50 μm; voltage: 17 kV; pressure injection: 2s; detection: absorption at 200 nm. Peak numbers correspond to Fig. 1.

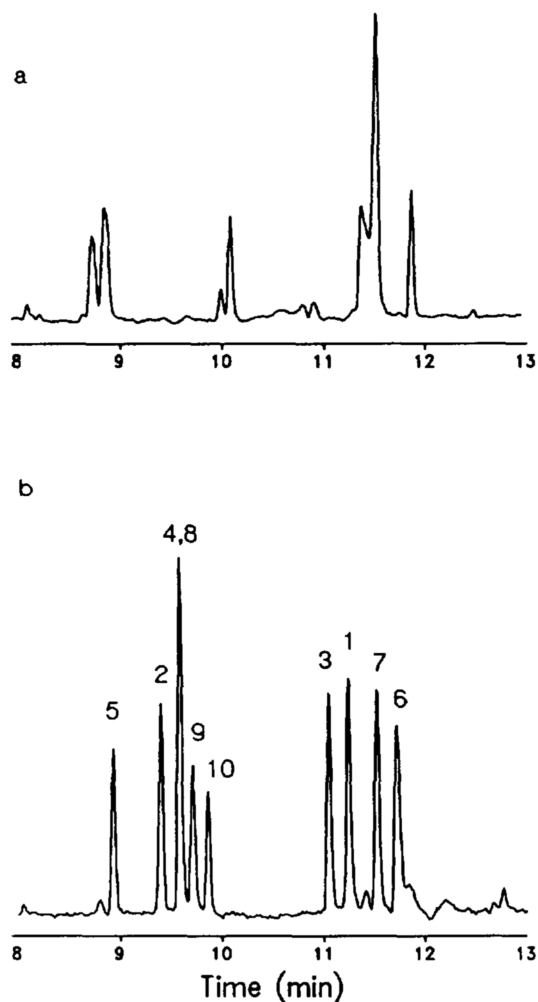

Fig. 3. MEKC of ten estrogens using bile salts. Buffers: 10 m*M* sodium borate (pH 9.2) containing (a) 100 m*M* sodium deoxycholate, and (b) 100 m*M* sodium cholate; capillary: 47 cm × 50 μm; voltage: 17 kV; pressure injection: 2 s; detection: absorption at 200 nm. Peak numbers correspond to Fig. 1.

separation of the estrogens was not successful (data not shown). This is because the estrogens did not form inclusion complexes with α-CD because its cavity is small compared to those of the β- and γ-CDs.

4. Conclusions

MEKC is a useful method for separating estrogens. With a 10 m*M* borate buffer (pH 9.2) containing 100 m*M* sodium cholate, nine of the ten tested estrogens were resolved. Buffers containing only SDS micelles allowed for partial separation of the estrogens. However, the addi-

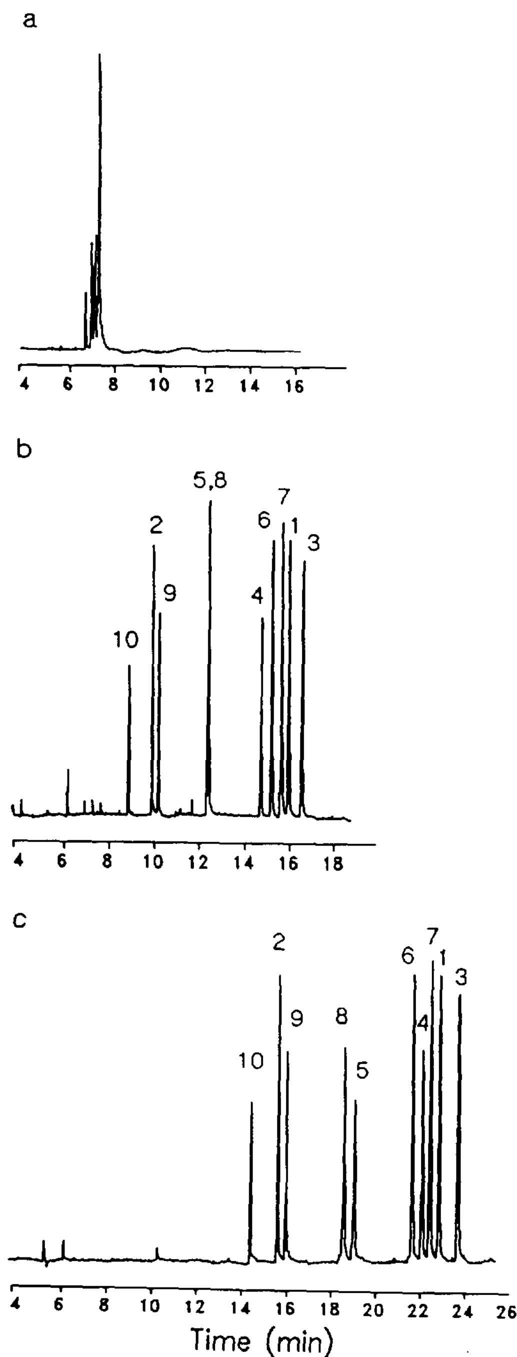

Fig. 4. Effects of organic solvents on MEKC of ten estrogens. Buffers: 10 mM sodium phosphate (pH 7.0) containing 50 mM SDS and (a) no organic solvent, (b) 15% acetonitrile and (c) 20% methanol; capillary: 47 cm × 50 μm; voltage: 20 kV; pressure injection: 2 s; detection: absorption at 200 nm. Peak numbers correspond to Fig. 1.

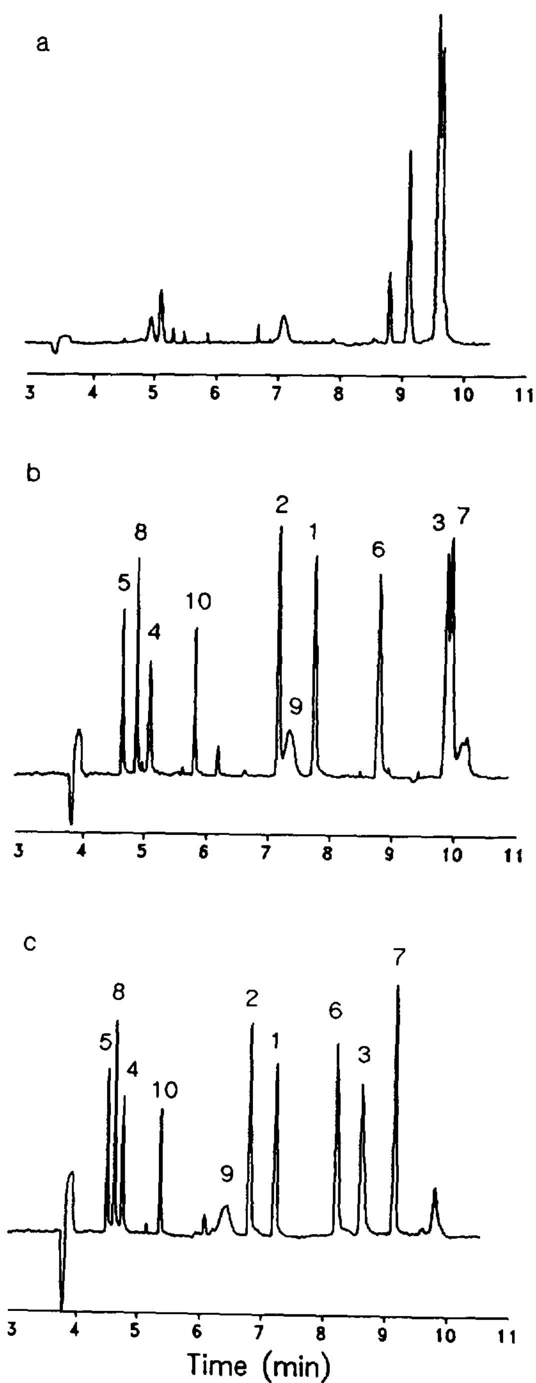

Fig. 5. Effects of cyclodextrins on MEKC of ten estrogens. Buffers: 10 mM sodium borate (pH 9.2) containing 50 mM SDS and (a) no CD, (b) 20 mM β-CD and (c) 20 mM γ-C; capillary: 47 cm × 50 μm; voltage: 17 kV; pressure injection: 2 s; detection: absorption at 200 nm. Peak numbers correspond to Fig. 1.

tion of modifiers to the SDS buffers, such as organic solvents or CDs, greatly improved the separations. Using either a 10 m*M* phosphate buffer (pH 7.0) containing 50 m*M* SDS and 20% methanol, or a 10 m*M* borate buffer (pH 9.2) containing 50 m*M* SDS and 20 m*M* γ-CD, all ten of the tested estrogens were separated. However, the CD-modified buffer is more desirable because it allows for a faster analysis time (ca. 10 min vs. 25 min). With UV absorption, the detection limits for the tested estrogens were ca. 2 μg/ml. An application of the described MEKC methods is the determination of estrogens in pharmaceutical dosage forms. For the determination of estrogens in biological samples, an extraction/concentration step or a more sensitive detection technique than absorption is needed and these are currently studied in our laboratory.

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