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# Separation of urinary estrogens by micellar electrokinetic chromatography

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**Abstract**

Urinary estrogen levels are important for monitoring the normal pregnancy process as well as for the diagnosis of reproductive diseases. 17β-Estradiol and estrone are maintained at very low concentrations in urine and, therefore, are difficult to determine using standard chromatography with UV detection. In the present study, we describe a potential method for the determination of urinary estrogens (estrone, estradiol and estriol) using a solid-phase extraction and rapid capillary electrophoretic (CE) separations. Micellar electrokinetic chromatographic (MEKC) analysis was optimized by evaluating the number of surfactants in a 5 m*M* borate–5 m*M* phosphate separation buffer, of which sodium cholate (75–90 m*M*) was found to be optimal. Changing the hydrophobic character of the separation buffer with organic additives had a significant effect on the resolution of the three estrogens and an internal standard (d-equilenin). The addition of an organic additive (20% acetonitrile) was found to be necessary for the resolution of all components of the mixture. Substitution with 20% methanol provided a similar separation with better resolution but at the cost of increased analysis time. Analysis of two extracted urine samples from 18-weeks and 21-weeks pregnant women showed that, with the present technology, CE can provide adequate resolution and superior speed, but the sensitivity limits attainable with the existing technology may limit its utility to the measurement of estriol and estrone.

## 1. Introduction

17β-Estradiol ($E_2$; dihydroxylated) is an essential hormone for maintenance of the female reproductive system and bone density [1]. Metabolism of $E_2$ to estrone ($E_1$, monohydroxylated) and estriol ($E_3$, trihydroxylated; 16α,17β) leads to the presence of these steroids in urine [2] (structures given in Fig. 1). The urinary $E_3$ concentration secreted by the placenta is known to gradually increase to a maximum concentration of approximately 1 mg/day (resultant concentration of 16 μg/ml) as a normal pregnancy progresses [3]. Interest in the measurement of estrogens in serum, saliva, urine and other biological fluid has intensified as a result of the numerous potential clinical applications. It has been reported that serum $E_1$ levels can provide an accurate marker for septic shock [4] and that a significant increase in the plasma $E_1$ level is indicative of myocardial infarction up to 3 months later [5]. Maternal serum $E_3$ is mainly used in prenatal screening as one of the markers for Down's syndrome [6–10]. Elevated

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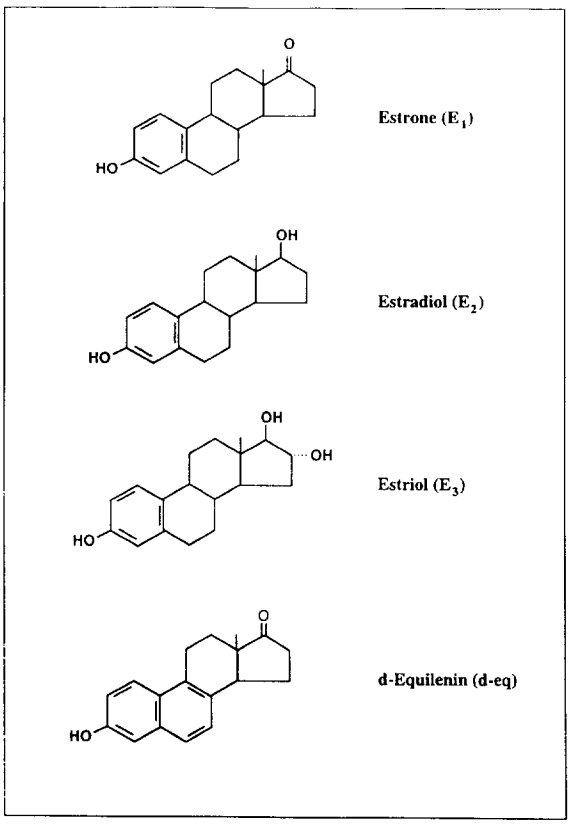


Fig. 1. The structures of estrogens and the internal standard, d-equilenin.

maternal serum $\alpha$-fetoprotein, very low unconjugated estriol, and very low human chorionic gonadotropin has recently been associated with fetal death [6]. Furthermore, considerable interest has been generated in the potential relationship between 17$\beta$-estradiol ($E_2$) in various tissues and body fluids, and the diagnosis of certain disease states. Amniotic $E_2$ significantly increases in women delivered prematurely [11] and decreases in alcoholic pregnant women [12]. $E_2$ concentration also increases in the cytosol of term placenta from hypertensive pregnant patients [13], and decreases in the plasma of patients with breast cancer [14,15]. Determination of $E_2$ can also differentiate the diagnosis of cystic ovarian tumors as functional (higher tissue $E_2$ levels) or nonfunctional (lower tissue $E_2$ levels) [16]. It has also been found that higher $E_2$ concentrations are present in brain tumor tissue in patients with malignant astrocytoma [15]. The determination of estradiol in synovial fluid has revealed that excessive synovial estradiol levels and elevated estradiol receptor binding may be involved in the development of knee osteoarthritis, particularly in postmenopausal women [17].

Urinary $E_2$ and $E_1$ concentrations are typically low relative to $E_3$ and fluctuate during gestation. Ishida et al. [18] reported that urinary $E_1$ and $E_2$ levels in 7–38-weeks pregnant women range from 0.013 to 1.22 mg and from 0.094 to 0.203 mg/24 h urine, respectively. While they are difficult to measure by standard liquid chromatographic techniques, many methods have been shown to be effective for determining urinary $E_3$ levels. They include radioimmunoassay [19], silver–silver chloride electrode detection (the conversion of these estrogens into electroactive nitro derivatives of estrogens, which are used for voltametric determination) [20], enzyme-linked immunoassay [21], HPLC with electrochemical detection [22] and fluorescence detection [23,24]. Only a few methods have been shown to be feasible for the measurement of $E_1$ or $E_2$, of which the most common methods are radioimmunoassay (RIA) [11,12,25,26] and enzyme-linked immunosorbant assay (ELISA) [27,28], and to a lesser extent, HPLC [29]. None of these methods are capable of determining $E_1$, $E_2$ and $E_3$ simultaneously, as a result of either $E_1$ interfering with $E_2$ and $E_3$ in electrical detection or the level of $E_2$ approaching the lower limit of detection of the HPLC. The most common method for measurement of estrogens is radioimmunoassay which suffers from several drawbacks. While sensitive, it is dramatically higher in cost, subject to 1–3% cross-reactivity and lacks sensitivity for urinary $E_2$. Similarly, fluorescent detection methods require extraction and are complicated by the requirement for derivatization schemes. In the current study, we detail the results of the first phase in the development of a simple, sensitive assay for urinary estrogens ($E_1$, $E_2$ and $E_3$): optimization of conditions for rapid separation using micellar electrokinetic chromatography. The MEKC separation was optimized through the evaluation of (1) different surfactants, (2) the concentration-dependence of

specific surfactants, and (3) organic solvent addition to the separation buffer. d-Equilenin was chosen as internal standard because it is a non-detectable metabolite in urine and had an extraction efficiency similar to the other three estrogens in this study. An estrogen standard mixture containing estradiol, estrone, estriol and d-equilenin, in a 1:1:1:1 concentration ratio, was used to identify and optimize the MEKC separation conditions.

## 2. Experimental

### 2.1. Materials

Borate acid, sodium tetra borate, sodium dodecyl sulfate, sodium hydroxide, 17β-estradiol, estrone, estriol and d-equilenin were purchased from Sigma (St. Louis, MO, USA). Zwittergent detergents, 3-10 detergent (N-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, DDAPS 3-10, aggregation number 41), 3-12 detergent (N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (DDAPS 3-12; aggregation number 55) were from Calbiochem (La Jolla, CA, USA). Sodium cholate, sodium taurocholate, sodium glycodeoxycholate, α-, β-cyclodextrin (CD) hydrates were from Aldrich (Milwaukee, WI, USA). Sodium phosphate was commercially obtained from Fisher Scientific (Fairlawn, NJ, USA). Acetonitrile (ACN) and methanol (MeOH) were HPLC grade and from Burdick and Jackson High Purity Solvents (Muskegon, MI, USA). $C_{18}$ Bond Elut cartridges (200 mg $C_{18}$; 3 ml bed volume) were from Varian (Harbor City, CA, USA). Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AR, USA).

### 2.2. Capillary electrophoresis instrumentation

Micellar capillary electrophoresis (CE) separations were carried out on a Beckman P/ACE 5510 system equipped with retrofittable UV and diode array detectors. This was interfaced with a 486/66 MHz IBM Valuepoint computer utilizing System Gold Version 8.1 software for control and data collection. Bare fused-silica capillaries having dimensions of 47 cm × 50 μm I.D. (40 cm to the detector) were used. Data collected with the scanning diode array detector was from 200 nm to 350 nm.

### 2.3. CE separation conditions

The separation buffer contained 5 m*M* borate–5 m*M* phosphate with surfactant and acetonitrile or methanol added at concentrations described in the figure legends. The optimum buffer for analysis of the urine extract was 5 m*M* borate, 5 m*M* phosphate, 75 m*M* cholate, and 20% methanol, pH 8.86. All buffers were made with Milli-Q (double deionized) water and filtered through a 0.2-μm filter (Gelman) before use. The standard method included: a 3-min prerinse with separation buffer; a separation at 28 kV under constant voltage with the inlet as the anode and the outlet as the cathode; a 1-min 0.1 *M* NaOH rinse and a 1-min water rinse followed by a 3-min separation buffer rinse. The capillary was thermostated at an extra-capillary temperature of 25°C. Detection was either at a single wavelength of 200 nm or was scanned from 200 to 350 nm with the diode array detector.

### 2.4. Sample preparation and urine extraction

The standard mixture of estrogens contained 250 μg/ml each of $E_1$, $E_2$, $E_3$ and d-equilenin (internal standard) in 100% methanol. In order that evaporation be minimized, all samples were kept in the freezer (−20°C) until used. Briefly, the urinary estrogen extraction was accomplished as follows: to hydrolyze the conjugated estrogens, 450 μl of concentrated hydrochloric acid was added to 3.0 ml of urine and heated on a heating block at 100°C for 60 min. The purple solution was cooled to room temperature and 50 μl of 100 μg/ml internal standard (d-equilenin) in methanol was added. A volume of 10 ml of methylene chloride was added to the above solution, vortex-mixed for 2 min and centrifuged for 5 min at 1500 *g* at 4°C. The aqueous phase (top layer) was removed by aspiration. An aliquot of 1 ml of the solution containing 0.1 *M*

sodium hydroxide and 0.1 *M* sodium bicarbonate was added to the organic phase, vortex-mixed and then centrifuged for 3 min at 1500 *g*. The aqueous phase was discarded. In a similar process, 1 ml of 8% sodium bicarbonate and 1 ml of distilled water were used to wash the organic phase. The organic phase was dried under nitrogen flow in a water bath of 45°C. The dried extract was reconstituted with 400 $\mu$l of 50% (v/v) methanol–water and loaded onto a $C_{18}$ Bond Elut cartridge which had been conditioned with 3.0 ml of water, acetonitrile and water prior to sample loading. An additional 0.5 ml of MeOH was used to rinse the sample vial and was also loaded onto the $C_{18}$ cartridge. The sample on the $C_{18}$ cartridge was washed with 2.0 ml of water (eluant discarded), 2.0 ml of 22% acetonitrile–water solution (eluant discarded), 1.0 ml of 30% acetonitrile solution (eluant collected —contained $E_3$), 1 ml of 40% acetonitrile solution (eluant discarded) and 1 ml of 55% acetonitrile–water solution (eluant collected —contained $E_1$, $E_2$ and d-equilenin). These two fractions were combined and dried under nitrogen at 45°C. The final extract was stored at −20°C until use. The dry extract was reconstituted with 15 $\mu$l of pure methanol and then injected onto the CE capillary immediately.

## 3. Results and discussion

Separation and quantification of urinary estrogens provide clinically relevant information about several diseases related to abnormal metabolism. The measurement of steroids is desirable since specimens are not only easy to obtain, but estrogens are relatively stable in this matrix. Estrogens are uncharged compounds with a structure consisting of a nonpolar phenanthrene ring with hydroxy functional group(s) (Fig. 1). As a result of their uncharged nature, these analytes are not amenable to separation by capillary zone electrophoresis (CZE) where resolution is based on charge-to-mass ratios. However, micellar electrokinetic chromatography (MEKC), one specific mode of CE, has been shown to be useful for the separation of uncharged analytes [30]. With the MEKC technique, surfactants are added to the running buffer at concentrations above the critical micelle concentration (CMC) and form micelles whose characteristics are dependent upon the surfactant used. Uncharged analytes interact with micelles, both through their surface and through micellization, and are separated by electrophoresis due to a differential partitioning into or interaction with the micelles. The mobility of any uncharged (or charged) analyte in MEKC strongly depends on the nature of the surfactant used. As a result, separation and detection results from a combination of the electrophoretic mobility of the micelle/analyte and the endoosmotic flow (EOF). Endoosmotic flow results from the positively-charged buffer ions which form a layer along the inner capillary wall due to the negative charge from silica. During electrophoresis the layer of cations is drawn to the cathode (negative electrode) and acts as a pumping mechanism to propel a bulk flow of fluid from the inlet to the outlet. This process of endoosmotic flow drives the movement of all molecules (positive, neutral and negative) toward the cathode.

The mobility of micelles and the binding capacity of solutes are strongly influenced by the nature of the hydrophobic moiety, charged head group and, perhaps, the counterion of surfactant-forming micelles. For example, SDS is a well-established anionic surfactant which forms spherical micelles (aggregation number 60) at a CMC of ca. 8 m*M* with a relatively hydrophobic interior and a hydrophilic exterior. These micelles have been shown to be extremely useful for the separation of non-polar analytes due to the very hydrophobic nature of the micelle. In contrast, sodium cholate is a bile salt with a planar steroid-like structure presenting a polar and non-polar face. It does not form spherical SDS-like micelles but instead, at a CMC of ca. 14 m*M*, forms secondary structural aggregates (aggregation number 2-10) that are held together primarily by hydrogen bonding. As a result of their different properties and the different selectivities that they bring to MEKC separations, a

series of surfactants were evaluated for their ability to affect the separation of the estrogens.

### 3.1. Effect of different surfactants

The surfactants that were evaluated included two cyclodextrins ($\alpha$ and $\beta$), two zwitterionic detergents (DDAPS 3-10 and 3-12) and the anionic surfactants, SDS, sodium glycodeoxycholate, sodium taurocholate and sodium cholate. Each of these were tested at no fewer than four concentrations above the CMC in 5 m*M* borate–5 m*M* phosphate buffer, pH 8.9. Since it was realized early in this study that the separation buffer required an organic additive to maintain adequate solubilization of the analytes, 20% acetonitrile was added to each of the tested buffers. Fig. 2 shows the electropherograms resulting from the CE separation of the estrogen mixture at a single concentration with six of the tested surfactants. Both of the zwitterionic detergents tested were ineffective in resolving the components of the estrogen standard mixture. Fig. 2A shows the separation obtained with 30 m*M* DDAPS 3-12 (CMC 2–4 m*M*) where all components of the mixture are seen to co-migrate with the EOF. Identical separations were observed with the shorter alkyl chain form, DDAPS 3-10 (not shown). The co-migration of all components with the EOF indicates that no significant interaction occurred between the estrogens and the micelle. Increasing the concentration of the DDAPS additives did not improve the separation. As shown in Figs. 2B and 2C, cyclodextrins at a concentration of 50 m*M* had a favorable effect on the separation of the estrogen mixture. The utility of cyclodextrins (CD) in CE has been increasing for various uncharged or chiral compound separations [31]. Cyclodextrins are oligosaccharides consisting of several D-( + )-glucopyranose units; $\alpha$ and $\beta$ designate CDs containing six and seven glucopyranose units, respectively. Both surfactants can enhance estrogen separation as a result of the hydrophobic nature of the open cavity in the CD molecule, which is capable of preferentially retaining or "guesting" structurally different estrogens. The outer part of the CD molecule is very hydrophilic in character. In a buffer containing 5 m*M* borate–5 m*M* phosphate and 50 m*M* $\alpha$-CD, $E_3$ is well resolved from $E_1$, $E_2$ and d-equilenin, indicating that these analytes are preferentially retained in the cavity of the $\alpha$-CD molecule. In this particular separation, a second internal standard, equilin, was added and it too was resolved from the other mixture components. The $\beta$-CD (50 m*M*) was found to be less effective than its $\alpha$-counterpart. The peaks are severely compressed and, as indicated by their migration close to the methanol peak (EOF marker), appear to interact poorly with the $\beta$-CD, possibly indicating that the larger cavity size allows a less effective interaction. While clear resolution of $E_3$ and $E_1$ was obtained, $E_2$ was not resolved from

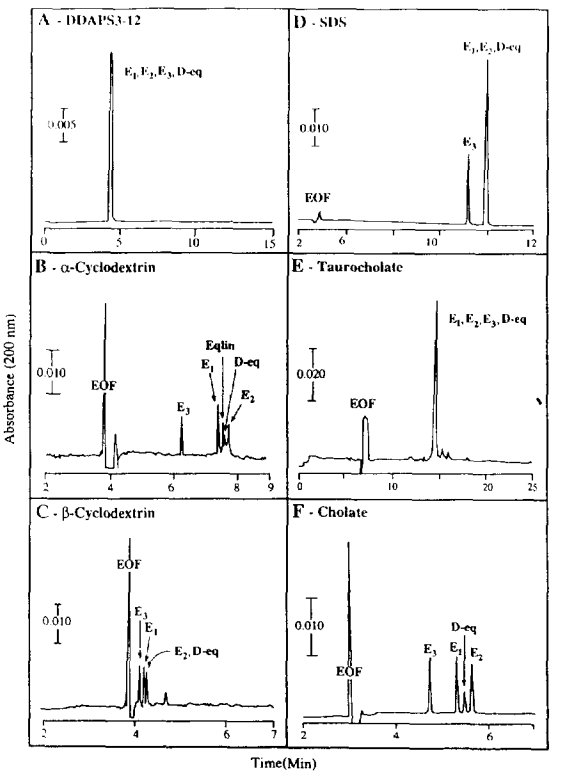


Fig. 2. The effect of type of surfactants on MEKC separation. The running buffers were as follows: (A) 30 m*M* DDAPS3-12, pH 8.4; (B, C) 50 m*M* $\alpha$- or $\beta$-cyclodextrin, pH 8.6; (D–F) pH 8.8, 150 m*M* SDS (D), 50 m*M* taurocholate (E) and 75 m*M* cholate (F). All separations were carried out at 30 kV. The system current associated with these separations were 13, 60, 68, 89, 42 and 79 $\mu$A, respectively.

the d-equilenin. Of the four anionic surfactants tried, SDS provided resolution of $E_3$ but did not allow for separation of the other mixture components ($E_2$, $E_1$, d-equilenin) (Fig. 2D). Taurocholate (50 m*M*) was found to be ineffective as a micelle-forming surfactant (Fig. 2E). Although the mixture components interacted with the micelle, this appeared to occur without any resultant preferential partitioning. Results obtained with glycodeoxycholate (data are not shown) were similar to those in Fig. 2E. The optimal separation (best resolution, shortest analysis time) was obtained with sodium cholate (Fig. 2F). Although an anionic detergent like SDS, sodium cholate is a bile salt with a planar steroid-like structure presenting a polar and non-polar face. It does not form spherical SDS-like micelles but instead, at a CMC of ca. 14 m*M*, forms secondary structural aggregates (dimers to octamers) that are held together primarily by hydrogen bonding. At a concentration of 75 m*M*, resolution of all components was obtained in less than 6 min with the best signal-to-noise (*S/N*) ratio at 200 nm.

It is worthy of mention that similar work has recently been carried out by Chan et al. [32]. In agreement with the results of the present study, cholate appeared to be the best surfactant for resolving $E_1$, $E_2$ and $E_3$ as well as for providing the best sensitivity with UV absorbance. In addition, Chan et al. [32] have shown that a mixture containing $E_1$, $E_2$ and $E_3$ and seven other estrogens was well resolved in 100 m*M* phosphate buffer containing 20 m*M* γ-CD and 20% methanol. d-Equilenin was not included in their standard mixture.

### 3.2. Effect of cholate concentration

In an attempt to optimize the separation of the estrogen mixture in a buffer containing sodium cholate, several buffer parameters were independently varied to determine the effect on the separation, beginning with the optimization of the buffer cholate concentration (Fig. 3). When sodium cholate was added to 5 m*M* borate–5 m*M* phosphate, pH 8.90, at concentrations slightly greater than the CMC, no resolution of

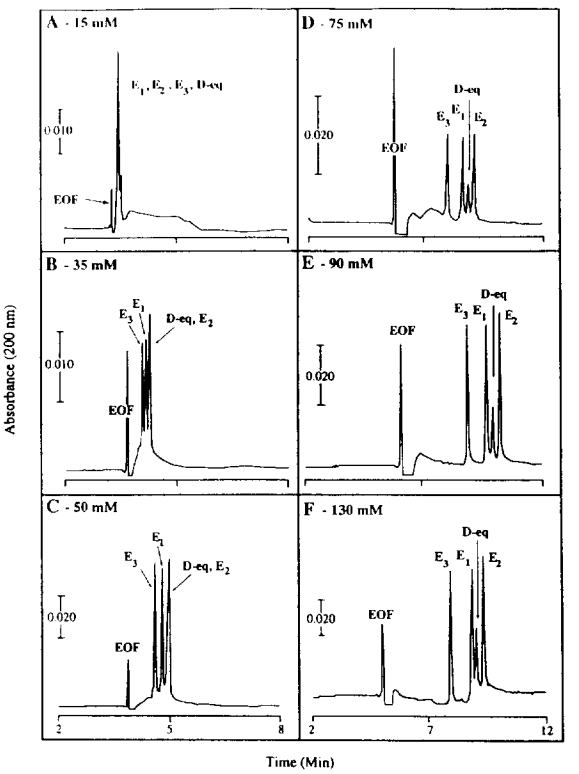


Fig. 3. The effect of buffer cholate concentration on separation at pH 8.66–8.90. Separation was carried out with 5 m*M* borate, 5 m*M* phosphate, 75 m*M* cholate, pH 8.6, and cholate concentrations of 15, 35, 50, 75, 90, and 130 m*M*, respectively, for A–F. The voltage was 28 kV and the system currents associated these separations were 21, 34, 34, 59, 68, and 94 μA, respectively.

the mixture components was observed. Under these conditions, little interaction of the estrogens with the micelle appears to have occurred as evidenced by migration of the peak close to the EOF (Fig. 3A). At 35 and 50 m*M*, increased interaction with the micelle occurred and some resolution was observed, although d-equilenin was not resolved from $E_2$. The optimum cholate concentration for separation of the estrogens was found to be in the 75–90 m*M* range. Assuming that the difference in mobility of each of the mixture components is due to the partitioning into or interaction with the micelle, it is clear that it is in this surfactant concentration range that the differences are maximized. At cholate concentrations greater than 90 m*M*,

all peaks were shifted to longer migration times without an enhancement in resolution (Fig. 3F). In fact, in separation buffer containing 130 m*M* cholate, the d-equilenin peak is slightly shifted into the $E_1$ peak, thus, impeding baseline resolution. At higher cholate concentrations (e.g. > 150 m*M*), the resolution of $E_1$, $E_2$ and $E_2$ was maintained but d-equilenin was no longer resolved from the $E_1$ peak (data not shown). It is obvious that, at higher concentrations, the differential interaction of $E_1$ and d-equilenin with the micelle is lost. These results not only suggest that, in the presence of different concentrations of cholate, the interaction between micelles and the individual estrogen mixture components is different, but that d-equilenin was slightly more sensitive to changes in the buffer cholate concentration.

### 3.3. Effect of acetonitrile and methanol as buffer additives

The addition of an organic co-solvent to a micellar solution has been shown to improve the separation of highly hydrophobic compounds that migrate near or with the micelles [33]. Fig. 4 shows the results of varying the concentration of ACN (added to separation buffer containing 5 m*M* borate–5 m*M* phosphate–75 m*M* cholate, pH 8.90) on the separation of the estrogen mixture. Optimal resolution of the mixture components was obtained with either 20% ACN or 50% ACN in the separation buffer. Two remarkable differences were apparent in these two separations. The first, and most obvious, was the increase in analysis time which was approximately doubled as a result of the organic additive repressing EOF [18]. In light of the fact that the goal of this study was to develop a rapid CE-based assay for urinary estrogens, the prolonged migration times associated with 50% ACN was thought to be undesirable. In addition, there was an accompanying increase in baseline noise which indirectly affects the sensitivity of the assay. Finally, there was an interesting change in selectivity. In the same manner that d-equilenin is differentially affected by changes in the cholate concentration, there is a dramatic change in

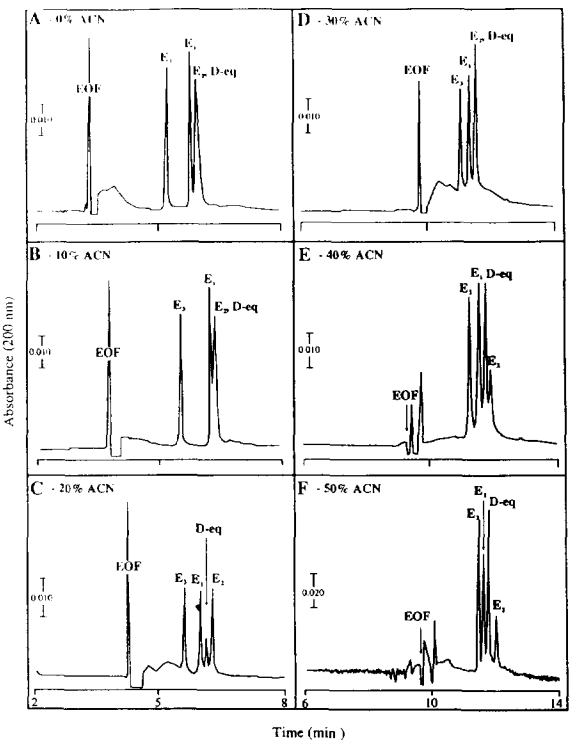


Fig. 4. The effect of buffer acetonitrile (ACN) on the separation of the estrogens. Separation was carried out in buffer containing ACN ranging from 0 to 50% (v/v) in 5 m*M* borate, 5 m*M* phosphate, and 75 m*M* cholate, pH 8.6. The voltage was 28 kV and the system currents associated these separations were 71, 69, 59, 54, 51, and 48 $\mu$A, respectively, for A–F.

the mobility of d-equilenin in response to the buffer ACN concentration. In 10% ACN, d-equilenin and $E_2$ have roughly the same mobility, while 20% ACN allows d-equilenin to interact with the micelles less effectively than $E_2$. In 30% ACN, the $E_1$, $E_2$ and $E_3$ peaks became somewhat compressed and the mobility of $E_2$ and d-equilenin equalized. At ACN concentrations greater than 30%, the mobility of d-equilenin actually overcomes $E_2$ as the slowest migrating species (i.e., the most interaction with the micelle) but, as mentioned earlier, the migration times now have become undesirably long from an assay development perspective.

Methanol, a solvent that is more polar than acetonitrile, was evaluated as a organic buffer additive since it was also used as a matrix

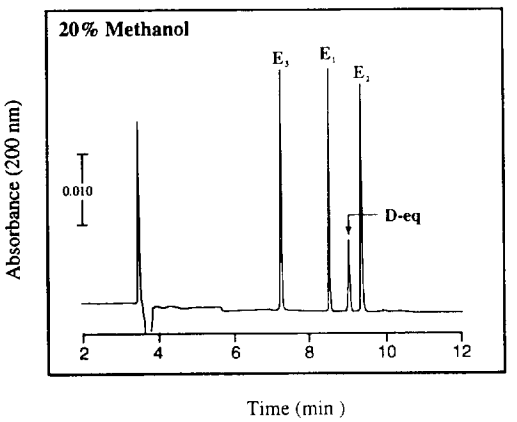


Fig. 5. The effect of buffer methanol (MeOH) on the separation of the estrogens. The separation was carried out in a buffer containing 20% MeOH (v/v), 5 m*M* borate, 5 m*M* phosphate, 75 m*M* sodium cholate at pH 8.70. The voltage was 28 kV and the system current associated with this separation was 47 μA.

(solvent) for the estrogen mixture. Only a single electropherogram is shown in Fig. 5 which was obtained in separation buffer containing 5 m*M* borate–5 m*M* phosphate–75 m*M* cholate, pH 8.86, and 20% methanol. A buffer containing 20% methanol provided better resolution than that obtained with 20% ACN, albeit with longer migration times (analysis was complete in < 10 min). However, while the time-frame for separation was approximately equal to that observed in the presence of 50% ACN, MeOH provided a significant improvement in peak shape (sharpening), and resolution improved over that observed with ACN. Calculating the resolution achievable with separation in 20% MeOH and 20% ACN showed that resolution was, indeed, superior in the presence of MeOH without adversely affecting the reproducibility (see Table 1). Attempts were made to decrease the analysis time by shortening the capillary length (from 47 to 37 cm, 50 μm I.D. maintained). However, while the analysis time was reduced to less than 7 min, it was associated with a marked tailing of the peaks.

Finally, Fig. 6 illustrates the potential utility of scanning diode array detection for identification of analyte peaks. Diode array detection from 200 to 300 nm showed that, under these separation conditions, the individual estrogens in a standard mixture have a significant absorbance maximum at 230 nm and a small but measurable absorption at 280 nm. This can be seen in the three-dimensional illustration given in Fig. 6A. As expected, the individual estrogens can not be differentiated based on their spectral properties. However, the internal standard, d-equilenin, which will eventu-

Table 1
Reproducibility of individual estrogen migration times

| Estrogen | Resolution | Mean MT (min) | C.V. (%) |
|---|---|---|---|
| *Cholate buffer, 20% ACN* | | | |
| $E_3$ | | 5.40 | 1.36 |
| $E_1$ | 3.97 | 5.76 | 1.73 |
| d-Eq | 1.54 | 5.90 | 1.69 |
| $E_2$ | 1.44 | 6.01 | 1.76 |
| *Cholate buffer, 20% MeOH* | | | |
| $E_3$ | | 9.14 | 1.31 |
| $E_1$ | 14.5 | 10.35 | 1.74 |
| d-Eq | 6.21 | 10.85 | 1.75 |
| $E_2$ | 3.79 | 11.17 | 1.73 |

CE conditions: the running buffer contained 5.0 m*M* borate, 5.0 m*M* phosphate, 75 m*M* sodium cholate and either 20% ACN or 20% MeOH. Voltage was 28 kV and the currents associated with the separations were 59 μA (20% ACN) and 46 μA (20% MeOH), respectively. MT = migration time; C.V. = coefficient of variation; resolution was calculated by $2(MT_2 - MT_1)/$(peak-width 2 at base line + peak-width 1 at base line).

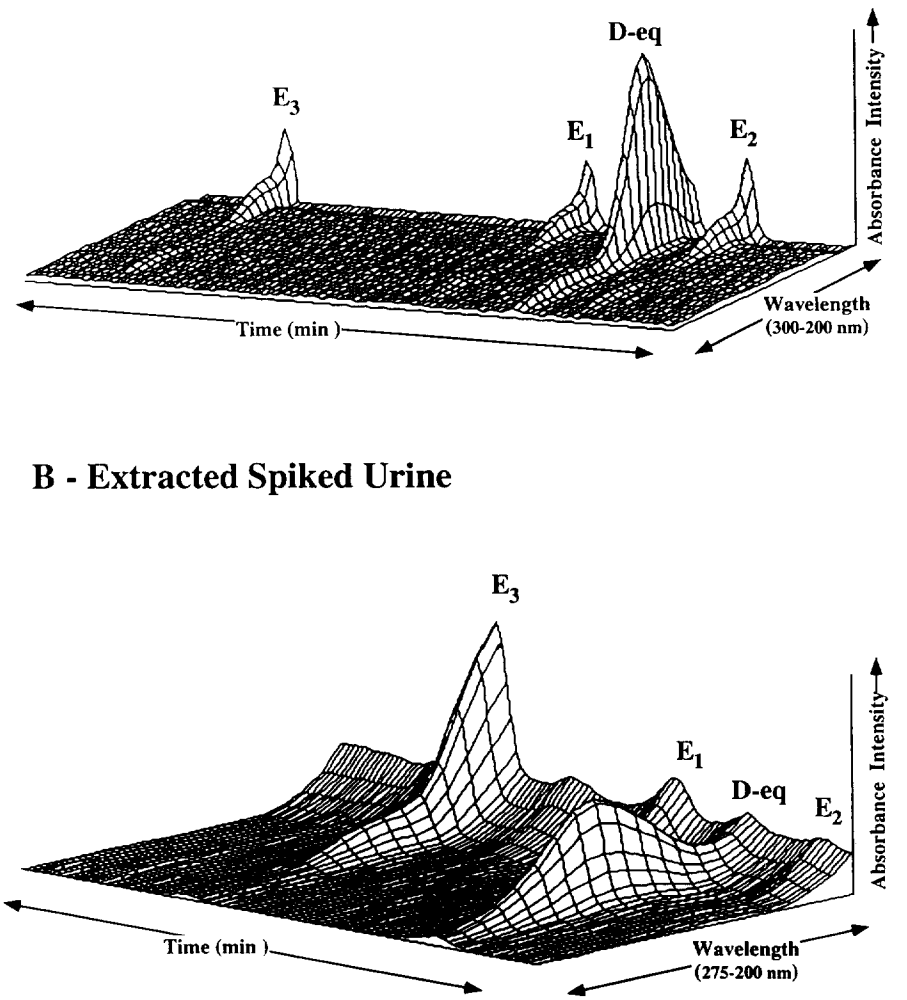


Fig. 6. Three-dimensional diode array electropherogram of the urinary estrogen separation. (A) Separation of standard estrogens and d-equilenin in methanol, concentration 250 $\mu$g/ml for each. (B) Separation of a urine extract (solubilized in methanol) from a 21-weeks pregnant woman. Both separations were carried out in the running buffer containing 5 m*M* sodium borate and 5 m*M* phosphate, 75 m*M* sodium cholate and 20% ACN at pH 8.69. Voltage was 28 kV and the system current associated with both separations was 64 $\mu$A. The diode array scans were from 200 nm to 300 nm.

ally be required for quantitation of the estrogens, can easily be identified by its strong absorption at 230 nm (Fig. 6A). When these compounds are spiked into urine and extracted for MEKC analysis, the unique spectra of the internal standard will allow for ease of internal standard identification (Fig. 6B).

### *3.4. Reproducibility of the assay*

Having optimized CE separation conditions with respect to separation buffer constituents (buffering components, surfactant, pH, ionic strength, etc.), capillary dimensions and voltage, the run-to-run migration time (MT) reproduci-

bility was evaluated. Table 1 shows the mean migration time, relative coefficient of variation (C.V.) for each of the estrogens separated and resolution between two peaks in 5 m*M* borate–5 m*M* phosphate–75 m*M* cholate in the presence of either 20% ACN or 20% methanol over 15 consecutive analyses ($n = 15$). In general, the C.V. (%) for migration time for each of the individual estrogens (and the d-equilenin) in either the ACN or MeOH system, were very good, not exceeding 1.8%. These values indicate that the only detriment to using the MeOH system is the extended separation time since the resolution is improved and the reproducibility is almost identical to that with the ACN system. It is not known at this time whether the increase in MT associated with the addition of MeOH will be problematic with respect to throughput. It is interesting that adequate MT reproducibility is attained with this system without specialized rinsing steps in the method. The use of a similar separation buffer system for the analysis of urinary drugs required an intermediary step wherein the capillary was rinsed with a 10-fold concentrated separation buffer prior to being rinsed with the regular separation buffer [34]. This emphasizes the analyte-dependent nature of migration time reproducibility in MEKC.

### *3.5. Extraction of estrogens from urine and MEKC analysis*

The ultimate goal of this study was to apply the optimal MEKC conditions to the analysis of estrogens in the urine of pregnant women as an indicator of fetal stress. There are a number of potential pitfalls associated with the analysis of estrogens in urine. One major problem is that a significant fraction of the estrogens present in urine are in the form of glucuronide and sulfate conjugates [35]. This, and the fact that a significant number of other steroids are also present in urine, can be circumvented with the implementation of an effective solid-phase extraction protocol. The extraction procedure described in Section 2 eliminates the majority of these interferences. Briefly, the first step in the extraction process functions to hydrolyze the conjugated estrogens using concentrated acid at elevated temperatures. All acidic components in the urine are uncharged after hydrochloric acid hydrolysis so that they are extracted into the methylene chloride phase. The use of a sodium bicarbonate–base wash allows for the extraction of acidic components which have large dissociate constants in the aqueous phase. The final stage of extraction eliminates unwanted steroids using water and acetonitrile prior to collecting estrogen fractions. The advantage of this procedure is that is provides a relatively "clean" estrogen extract for MEKC analysis.

The optimized MEKC system was used to analyze the extracted urine obtained from 18- and 21-weeks pregnant women who were presumed to have detectable estrogen levels, especially $E_3$ and $E_2$. Fig. 7 shows the results of separation in 5 m*M* borate–5 m*M* phosphate–75 m*M* cholate–20% MeOH of the extracted urine from an 18-weeks pregnant woman. Fig. 7A shows the resultant electropherogram when the urine extract is co-injected with the estrogen standard mixture. A comparison of this with the electropherogram in Fig. 7B, which lacks the co-injection of the estrogen standards, allows for identification of the extracted components. $E_3$ is easily identified and a clearly identifiable signal is obtained for $E_1$ and the internal standard. However, the signal in the region where $E_2$ is expected is barely detectable. At this time, there is no means of ascertaining whether the small signal observed is $E_2$ or some other co-extracting contaminant. This indicates that while the CE-based method provides a rapid assay with adequate resolution of the desired estrogens, the sensitivity limit of the existing system may not be adequate for detection of the least abundant estrogen, $E_2$.

## 4. Conclusions

As the first phase in the development of a simple and sensitive CE-based assay for urinary estrogens, we have defined the conditions for the rapid analysis of a urine extract by micellar electrokinetic chromatography. The optimal res-

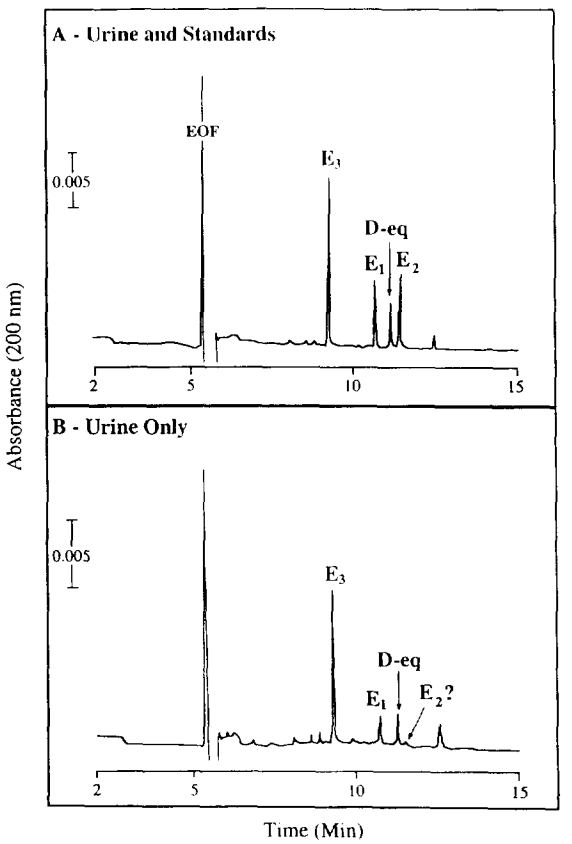


Fig. 7. Electropherograms of urinary estrogen separation. (A) Urine extract (from an 18-weeks pregnant woman) co-injected with the standard mixture of estrogens and d-equilenin. (B) The same urine extract as that shown in (A) without co-injection with standards. Both separations were carried out in the running buffer containing 5 m*M* sodium borate and 5 m*M* phosphate, 75 m*M* sodium cholate and 20% MeOH at pH 8.70. Voltage was 28 kV and the system current associated with both separations was 46 μA.

olution of $E_1$, $E_2$ and $E_3$ was achieved in a separation buffer containing 5 m*M* borate–5 m*M* phosphate–75 m*M* cholate and either 20% methanol or 20% ACN. The power of capillary electrophoretic approaches for providing rapid analyses is well illustrated in this study since resolution of the three estrogens and an internal standard was achieved in less than 7 min. Furthermore, the resolving power of this technique is highlighted by the resolution of two compounds ($E_1$ and d-equilenin) which are identical in charge and differ only by two double bonds in the B-ring. While further work is required for this to be useful for the quantification of urinary estrogens in a CE-based assay, this work identifies the potential utility of CE for the clinical analysis of steroids in urine. Further work is underway for enhancing the sensitivity of CE for the separation and detection of urinary estrogens. One of the approaches involves the exploitation of solid-phase extraction–capillary electrophoresis (SPE–CE), wherein analytes are concentrated on-capillary with a reversed-phase packing. This technique has already been shown to be useful for the analysis of analytes at concentrations well below the detection limit of standard CE [36,37]. The second phase in the development of this assay for clinical use will involve implementation of the SPE–CE technology for the detection of urinary estrogens.

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