Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 728–735

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Application of simple on-line sweeping sample concentration technique coupled micellar electrokinetic chromatography for simultaneous analysis of estrogen and androgen epimer

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ARTICLE INFO

Article history:
Received 7 January 2011
Received in revised form 14 July 2011
Accepted 21 July 2011
Available online 28 July 2011

Keywords:
Androgen
Estrogen
Epimer
On-line sweeping-MEKC
Saccharomyces cerevisiae
Stereoselective reduction

ABSTRACT

A reliable, convenient, and sensitive on-line sweeping-MEKC sample concentration technique has been applied for the simultaneous separation of six steroids including two pairs of epimer with 10 mM phosphate buffer (pH 7.0) that contains 80 mM sodium dodecyl sulfate (SDS), 14 mM β-cyclodextrin (β-CD), and 4% (v/v) methanol. The column length was 105 cm (effective length, 90 cm). Samples were hydrostatically injected for 600 s. The separation was performed at ambient temperature under an applied voltage of 25 kV. The external standard calibration curves of the six steroidal hormones proved good linearity (r^2 = 0.9785–0.9941) within the concentration range 0.025–1.0 μg mL^{-1}. The limit of detections of the on-line sweeping-MEKC with the ultraviolet detector at 220 nm for estrone, α-estradiol, β-estradiol, androstenedione, epitestosterone, and testosterone were 10, 24, 28, 53, 73, and 11 ng mL^{-1} and were 240, 125, 93, 47, 32, and 200 times more sensitive than MEKC, respectively. The *Saccharomyces cerevisiae* mediated simultaneous stereoselective reduction of estrone and androstenedione exhibited a 100% stereoselectivity toward β-estradiol and testosterone. The accuracy and precision achieved for the spiking experiments of the sweeping-MEKC were 95–98% and less than 3.8% (RSD), respectively.

1. Introduction

Endocrine hormones produced in the body control growth, metabolism, reproduction, and many other functions of body and mind. Estrone and estradiol are called estrogen and are two important female sex hormones that control female sexual functions, such as the menstrual cycle, the development of breasts, and other secondary sexual characteristics. Testosterone is a male sex hormone called androgen and is responsible for the development of sex organs and for the secondary sexual characteristics. Both estrogens and androgens belong to steroid hormones. Recently, estrogens have been used for the disease treatment such as breast cancer [1–3] and androgens have been used for anti-bone loss [4] and taken by athletes to strengthen their body [5]. Thus they are important in the applications of pharmaceutical industry and medical diagnoses.

Many analytical methods have been developed for the determination of estrogens and androgens such as immunoassay [6,7], gas chromatography–mass spectrometry (GC–MS) [5,8,9], and high-performance liquid chromatography–mass spectrometry (LC–MS) [10–12]. The method of micellar electrokinetic chromatography (MEKC) [13], combining the electrophoretic and chromatographic properties of separation, uses micelles in solution as carriers for separation of compounds in a capillary tube under an applied electric field. The MEKC extends the capillary electrophoresis (CE) separation for neutral analytes and has the advantages of using small sample and simple instrumentation.

The micelles formation in MEKC is an anionic surfactant and the most commonly used one was sodium dodecyl sulfate (SDS). The pseudostationary phases of SDS [14,15] are only apparent due to the stability of the micelles. However, SDS does not have the ability to distinguish stereoisomer. In order to separate the stereoisomer (or epimer) of estrogens or androgens, some stereoisomer selectors must be added to the electrolyte to increase the selectivity for stereoisomer and α-, β-, and γ-cyclodextrin of different molecular sizes was the most often used stereo-selector *via* the formation of inclusion complexes by the host-guest mechanism [16–18].

However, the low sensitivity of UV detection caused by the small injection volume and narrow optical path length should also be overcome to make it suitable for trace estrogen and androgen stereoisomers analysis. Since estrogens and androgens lack native fluorescent property, a derivation procedure is necessary before fluorescence detection [19–21], and this inconvenience makes the fluorescence detection less widely used than the UV

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doi:10.1016/j.jpba.2011.07.024

detection. Although many CE–MS methods [22,23] have been rapidly developed and employed to increase the detection sensitivity, they subject the problems of expensive instrumentation and questionable separation for stereoisomer. Therefore, several major on-line sample preconcentration techniques [24] have been devised, such as dynamic pH-junction, transient isotachophoresis, electric field-enhanced sample stacking, and sweeping. Among the four techniques, sample stacking and sweeping [25,26] are suitable for concentrating neutral compounds for MEKC. Except for cases that the sample contains both neutral and ionic analytes, sweeping is superior to stacking in MEKC in terms of simple and convenient because it only relies on how the pseudostationary phase enters the sample solution zone (nonmicelle buffer) and sweeps (pick and accumulation) the analytes.

The aim of this paper is to develop an on-line sweeping sample concentration technique coupled MEKC for the simultaneous separation of estrogens and androgens including the epimers, "α- and β-estradiol" and "epitestosterone and testosterone," with the stereo-selector β-cyclodextrin. This technique was further applied to the trace analysis of yeast *Saccharomyces cerevisiae* catalyzed stereo-selective reduction culture of estrone and androstenedione.

2. Materials and methods

2.1. Chemicals

Reagent grade KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, D-(+)-glucose, and methanol of LC grade were all obtained from Merck (Darmstadt, Germany). Reagent grade $FeSO_4$ was bought from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Reagent grade estrone ($C_{18}H_{22}O_2$, MW 270.4), α-estradiol ($C_{18}H_{24}O_2$, MW 272.4), β-estradiol ($C_{18}H_{24}O_2$, MW 272.4), androstenedione ($C_{19}H_{26}O_2$, MW 286.4), epitestosterone (17α-hydroxy-4-androsten-3-one, $C_{19}H_{28}O_2$, MW 288.4), and testosterone (17β-hydroxy-4-androsten-3-one, $C_{19}H_{28}O_2$, MW 288.4) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Reagent grade Na_2HPO_4 and NaH_2PO_4 and β-cyclodextrin (β-CD, 98%) were bought from Sigma (St. Louis, MO, USA). Reagent grade NaOH, sodium dodecyl sulfate (SDS), and anhydrous $MgSO_4$ were supplied by Showa (Tokyo, Japan). HCl (37%) was the product of Riedel-deHaën (St. Louis, MO, USA). Reagent grade ethylacetate was got from Pharmco (Brookfield, USA). Freeze dried *S. cerevisiae* CCRC 21443 was bought from the Culture Collection Research Center of Food Industry Research and Development Institute (Hsinchu, Taiwan). High purity water (18.2 MΩ) was purified from tap water which was distilled once and further treated with EASYpure® II RF/UV ultrapure water system (Barnstead International, Dubuque, IA, USA).

2.2. Instruments and equipment

The CE system of gravity manual injection was equipped with a high voltage power supply of 30 kV maximum output voltage (Gamma High Voltage Research, Inc., RR30-2R, Florida, USA) and an ultraviolet (UV) detector (Rainin, Dynamax, UV-C, Michigan, USA) at a wavelength 220 nm. The wavelength selected was the intermediate value of the two maximum absorption wavelengths, 210 nm and 240 nm, for estrogen and androgen, respectively. The signal processing and data acquisition and processing were achieved by a PC through a multichannel interface (Agilent Interface 35900E, Waldbronn, Germany) and Agilent ChemStation software. Uncoated fused-silica capillaries (50 μm i.d. × 365 μm o.d. × 105 cm length with 90 cm effective length) were purchased from Polymicro Technologies, Inc. (Phoenix, AZ, USA). The whole CE instrument without any specific temperature control system was located in an air-conditioned laboratory which was usually controlled at 25 °C in the summer but the separation of steroids was under ambient temperature in the winter.

Prior to the first use of the capillary, the capillary must be conditioned to keep it in an optimum state which is treated with the following procedures: (1) flush with methanol for 20 min, (2) flush with 1.0 M HCl for 20 min, (3) flush with purified water for 4 min, (4) flush with 1.0 M NaOH for 20 min, (5) flush with purified water for 4 min, and (6) flush with background solution for 20 min. Then, during the sample analysis with either MEKC or sweeping-MEKC, the capillary is conditioned by the following sequence before sample loading and injection to ensure run-to-run reproducibility: (1) flush with 1.0 M NaOH for 5 min, (2) flush with purified water for 7 min, and (3) flush with background solution for 10 min.

2.3. The optimal conditions of MEKC analysis

The electrolyte of 1 mL 5 mM phosphate buffer (pH 7.0), 0.2883 g (50 mM) anionic surfactant SDS, 2% (v/v) methanol, and 18.6 mL deionized distilled water was used for the initial MEKC separation of the six standard steroids (100 ppm each) at an applied voltage of 20 kV [27]. The manual sampling time was 3 s and the separation temperature was under 25 °C. The UV detection wavelength was set 220 nm. However, the borate buffer (11, 17.5, 35 mM) has been surveyed by these separation conditions for its suitability for the separation of the six steroidal hormones.

At pH 7.0, four different phosphate buffer concentrations, 10, 15, 20, and 40 mM, have been tested to investigate their effect on the extent of peak tailing. Since no signal was detected for the buffer concentration below 10 mM, the buffer concentration of 10 mM was the lowest investigation concentration. In addition, the pH of phosphate buffer at 9.0 and 10.0 and with different buffer concentration has also been tested and the results have been compared with that at pH 7.0 to see the effect on analysis time and separation efficiency.

Twenty millimolar (0.5524 g) of the stereoisomer selector β-CD was first added to the above buffer solution to check the separation efficiency of β-CD for the six steroids, then the optimal concentration of the β-CD was tested for the concentration of 9, 10, 11, and 12 mM under the same operation conditions as above.

Since the addition of an organic modifier not only affects the quantity of electroosmotic flow (EOF) but also decreases the hydrophobic interaction between the analyte and micelle. In this study, methanol or acetonitrile has been used for the organic modifier. However, acetonitrile dissolves the outer polymer protective coat to make the inner capillary fragile and easily broken. Since methanol does not have this capability, methanol was chosen as the organic modifier. The separation effect of methanol for the six steroids was studied by using four kinds of methanol volume proportion in BGS (4%, 6%, 8%, or 10%). Since the six steroids will separate from the solution and block the capillary at 2% volume proportion of methanol in BGS by the very low water solubility of the steroids, 4% methanol volume proportion in BGS was the lowest concentration for testing.

During the electro-migration, the steroids distribute themselves between the buffer solution and the SDS micelles. Therefore, the concentration of SDS used for the separation is utmost important. The SDS concentration of 30, 35, 40 and 50 mM was tested for their effect on separation.

Since the applied separation voltage affects the separation time and the electric dispersion (the peak tailing), five kinds of applied separation voltage *viz.* 15, 17.5, 20, 22.5 and 25 kV were tested for the two effects.

2.4. Preparation of buffer solution

Phosphate buffer (100 mM) solution (pH 7.0) was prepared by dissolving 0.70 g NaH_2PO_4 and 0.60 g Na_2HPO_4 in 100 mL purified water and the pH of the buffer was monitored by a pH meter during the preparation. The background solution (BGS) was prepared by dissolving a suitable amount of SDS and β-CD in 0.8 mL methanol to bring them to the desired concentration, then incorporating this SDS/β-CD mixture into 2 mL phosphate buffer (pH 7.0), and finally adding 17.2 mL purified water to make a 20 mL 10 mM phosphate buffer. This BGS was ultrasonically treated for 30 min and rested for 60 min to wait for the disappearance of bubbles.

2.5. Simultaneous separation of the estrogen and androgen by MEKC and sweeping-MEKC

For MEKC analysis, the estrogen and androgen standards were weighed separately and dissolved in methanol to obtain the desired concentration. After the capillary was conditioned as described previously, the sample vial was raised to a height of 30.0 cm relative to the exit of the capillary to hydrostatically inject the sample for 3 s. The capillary inlet was then quickly put in the BGS, and the MEKC analysis was triggered by an applied voltage. The separation was progressed at ambient temperature.

For sweeping-MEKC analysis, the estrogen and androgen standards were weighed separately and dissolved in 1 mL methanol, 2 mL phosphate buffer (10 mM, pH 7.0), and 17 mL water to obtain the desired concentration. The sample injection procedure is the same as described in MEKC except that the sample was hydrostatically inject for 600 s. The difference between MEKC and sweeping-MEKC procedure is that test analytes in sweeping-MEKC are prepared in a matrix of lower buffer concentration than that of BGS but devoid of SDS and β-CD. Therefore, after injection and separation-voltage applying, test analytes in the sample zone in between the two BGS zones of lower conductivity move quickly to the interface where test analytes can be picked-and-accumulated (swept) by the reversed-migration anionic SDS.

2.6. Yeast mediated stereoselective reduction of estrone and androstenedione

Yeast *S. cerevisiae* grown and stored on agar slant was further inoculated and grown in 100 mL growth medium (1.5 g KH_2PO_4, 2.9 g K_2HPO_4, 1.3 g $(NH_4)_2SO_4$, 1.8 g $MgSO_4{\cdot}7H_2O$, 0.0175 g $CaCl_2{\cdot}2H_2O$, 0.1 mL (1.25%, w/v) $FeSO_4$, 20.0 g D(+)-glucose in 1.0 L water) for mass production. The incubation was carried out at 30 °C with a shaking rate of 150 rpm for 2 days. The 100 mL grown cell culture was transferred to the 3 L fermentor (Biotop, BTF-A3L, Taichung, Taiwan) that contained 0.9 L of the same growth medium for further cell growth. The cell culture was agitated at a stirring rate of 150 rpm accompanied with air bubbling through at a flow rate of 1 L min^{-1}. The temperature was controlled at 30 °C and the pH of the culture was controlled at 7.0 with a pH controller. The cell growth period was 2 days. Then, 1 mg estrone and 1 mg androstenedione dissolved in 10.0 mL absolute ethanol were directly added into the grown cell culture. The reaction was then performed at pH 5.0, a temperature of 30 °C, a stirring rate at 150 rpm, but without bubbling air through the system for 5 days. The scheme for yeast mediated simultaneous reduction of estrone and androstenedione to produce their corresponding α- and β-estradiol and testosterone and epitestosterone is shown in Fig. 1.

2.7. Pretreatment of cell culture

As the reaction was stopped, 20 mL cell culture was extracted by ethylacetate. The extract was dried with anhydrous $MgSO_4$ and further treated with vacuum evaporization to complete dryness. Afterwards, 1 mL methanol, 2 mL phosphate buffer (10 mM, pH 7.0), and 17 mL water were used to dissolve the completely dried extract. Then, the extracted cell cultures were loaded and analyzed by sweeping-MEKC according to the procedure described in previous section.

2.8. Quantitative analysis

The quantitative method for the simultaneous analysis of the six steroidal hormones in the cell culture with MEKC and sweeping-MEKC was the external standard calibration method. The linear concentration range of the six analytes used for the standard calibration curve was 0.8–100.0 μg mL^{-1} for MEKC and 0.025–1.0 μg mL^{-1} for sweeping-MEKC. The slope and the *y*-intercept of the best linear calibration curve were found by the least-square regression method with Excel software.

Since the limit of detection (LOD) of an analyte in instrumental analysis is defined as the three times of the ratio of the signal (*S*) to its noise (*N*) at its lowest concentration (LOD = 3*S*/*N*), one of the methods for obtaining the LOD is the use of the standard calibration curve constituted from the five lowest standard concentrations including the background solution (0 μg mL^{-1}) [28]. The limit of quantification (LOQ) for a specific analyte by a detector is, therefore, calculated at a higher level of quantitative reliability by LOQ = 10 × LOD/3.

The precision of the measurement for the analysis was estimated from the relative standard deviation (RSD) of the determined concentration with five repeated measurements of the cell culture. The analysis accuracy was estimated by the percentage recovered of the spike experiment that a certain amount of the standard was added to the cell culture. The percentage of recovery was calculated by comparing the recovered amount of standard with the spiked amount of standard.

3. Results and discussion

3.1. The optimal conditions of MEKC analysis

The separation time for six steroids with the use of borate buffer (pH 9.2) at 11, 17.5, and 35 mM are all longer than those with 10 mM phosphate buffer (pH 7.0). In addition, the separation between α-estradiol and estrone is poor and there are peak tailing for the six peaks with the borate buffer.

To see the effect of phosphate buffer concentration (10, 15, 20, 40 mM) on separation efficiency at pH 7.0, we found there was no improvement of the peak tailing at higher buffer concentration; in the meantime a prolonged analysis time was observed. At lower buffer concentration, only a small extent of improvement for peak tailing was found. However, if the buffer concentration was too low, the current produced will be too low to be used for analysis. Because no signal was detected for the buffer concentration below 10 mM, the buffer concentration of 10 mM was used as the optimal buffer concentration.

At pH 10.0, the separation of the six steroids was not ideal for phosphate buffer concentration at either 10 mM or 35 mM as compared with 10 mM phosphate buffer at pH 7.0. At pH 9.0, varying the buffer concentration (11, 17.5, 35 mM) and the applied voltage (15, 17.5, 20 kV), respectively, the results of separation for the six steroids show that the best separation exists for 17.5 mM buffer concentration and an applied voltage of 17.5 kV. However, the resolution between α-estradiol and estrone is poor and there are peak tailings for all six peaks as compared with the phosphate buffer at pH 7.0. Therefore, the optimal buffer pH is 7.0.

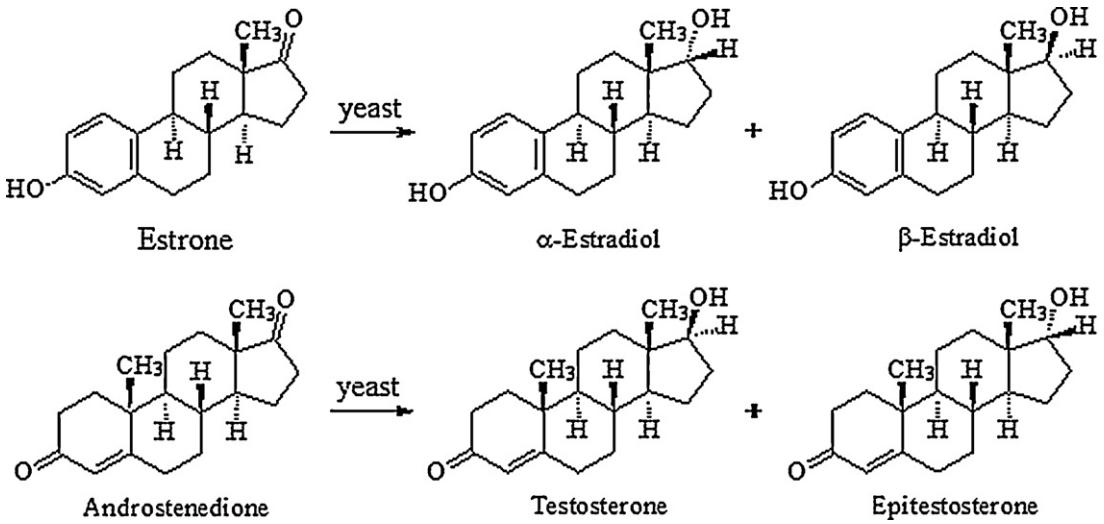

Fig. 1. The reaction scheme of *S. cerevisiae* mediated simultaneous stereoselective reduction of estrone and androstenedione.

Without the stereoisomer selector β-CD, the separation for the six steroids was inefficient, particularly, for the two pairs of epimer (α- and β-estradiol and epitestosterone and testosterone), due to the similarity in molecular structure, stereo-structure, and molecular size. The similarity of the six steroids makes them possess similar affinity with the SDS molecule. The results showed that 10 mM β-CD produced the best resolutions for the six steroids, therefore, the β-CD concentration of 10 mM was used for the rest of the MEKC analyses.

The results of the separation effect of the organic modifier methanol for the six steroids show that the separation time of the six steroid standards increases with an increase of the volume proportion of methanol in the BGS. The cause should be due to the suppression of EOF by methanol. Besides, the decrease of buffer viscosity by an increase of methanol proportion in BGS causes more serious peak tailing (electric dispersion). Thus, 4% methanol volume proportion in BGS was selected as the optimal value.

Experimental results show that the individual migration time for each of the six steroids running with different concentrations of SDS did not change substantially, however, the peak area of the six steroids varied significantly with different SDS concentrations. At 40 mM SDS, except for epitestosterone the peak area of the five steroids reached a maximum. The greatest peak area of epitestosterone (16.3 mAU × s) was in the run with 35 mM SDS, and the difference as compared with the peak area of epitestosterone at 40 mM SDS (15.9 mAU × s) was insignificant. Therefore, 40 mM SDS was used in later experiments.

Although an increase of applied voltage causes an increase of EOF, a decrease of the migration time of steroids, results indicate that the effect of the reduced migration times with an increase of the applied voltage on the separation among the six steroids was not significant. Therefore, the separation voltage of 25 kV was used for later experiments to give a shorter analysis time. The MEKC

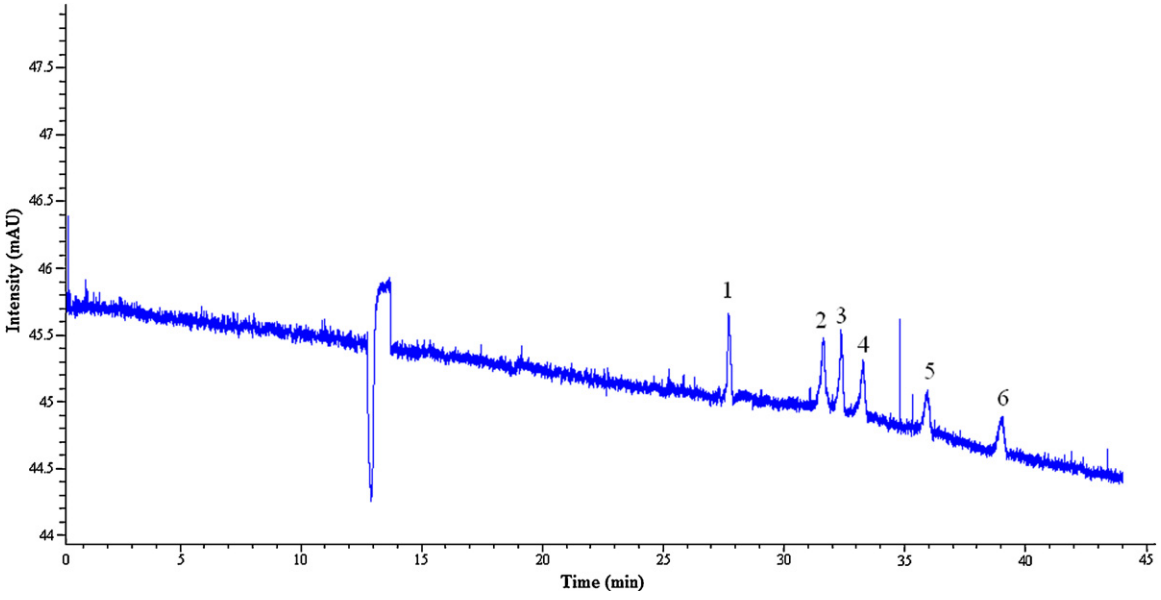

Fig. 2. Electropherogram of MEKC for the six steroidal hormones. Micelle solution: 2 mL 10 mM phosphate buffer (pH 7.0), 40 mM SDS, 10 mM β-CD, 0.8 mL 4% (v/v) methanol, and 17.2 mL deionized distilled water. Column length: 105 cm (effective length, 90 cm). Column temperature: ambient. Applied voltage: 25 kV. Siphonal height: 30 cm. Injection time: 3 s. UV detector: 220 nm. Peak 1: β-estradiol, peak 2: α-estradiol, peak 3: estrone, peak 4: testosterone, peak 5: androstenedione, and peak 6: epitestosterone. Sample concentration: 50 μg mL^{-1} of each standard sample dissolved in methanol.

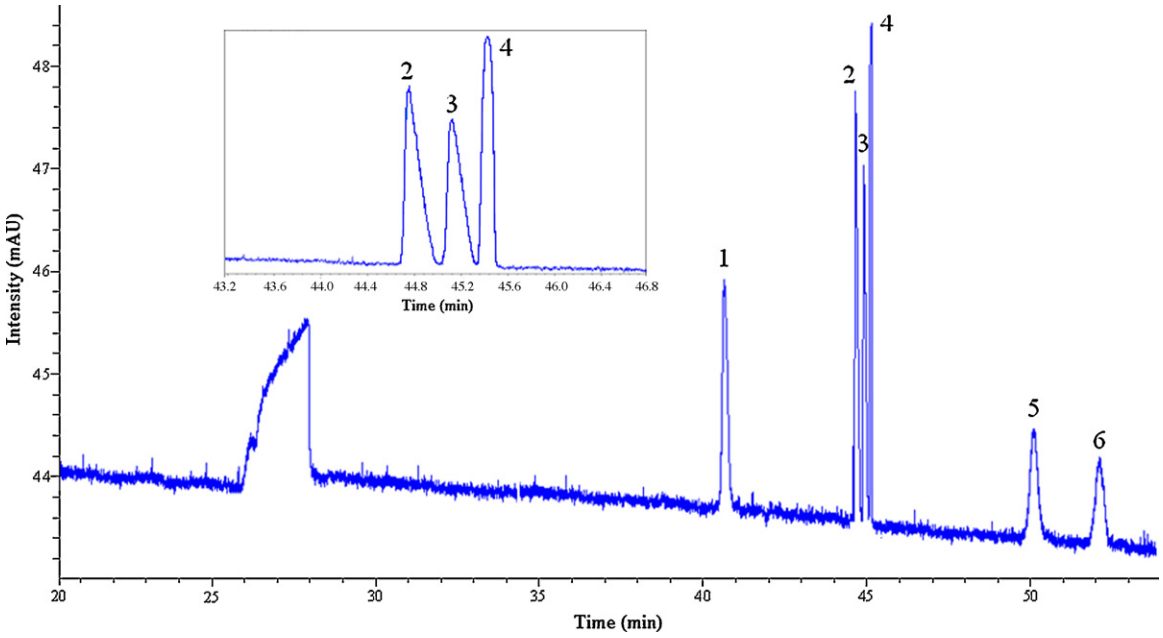

Fig. 3. Electropherogram of on-line sweeping-MEKC for the six steroidal hormones. Micelle solution: 2 mL 10 mM phosphate buffer (pH 7.0), 80 mM SDS, 14 mM β-CD and 0.8 mL 4% (v/v) methanol, and 17.2 mL deionized distilled water. Column length: 105 cm (effective length, 90 cm). Column temperature: ambient. Applied voltage: 25 kV. Siphonal height: 30 cm. Injection time: 600 s. UV detector: 220 nm. Peak 1: β-estradiol, peak 2: α-estradiol, peak 3: estrone, peak 4: testosterone, peak 5: androstenedione, and peak 6: epitestosterone. Sample concentration: 1 μg mL^{-1} of each standard sample dissolved in phosphate buffer.

separation for the six steroids with the obtained optimal conditions is shown in Fig. 2.

3.2. The on-line sweeping-MEKC technique

Although the technique of MEKC was able to simultaneously separate the six steroids including the two pairs of epimer successfully, the detection limit was about 7.5 μg mL^{-1} for the six steroids. Therefore, the on-line sweeping-MEKC sample concentration technique was employed to enhance the UV detector sensitivity. Initially, the on-line sweeping MEKC was performed with the optimal separate conditions of MEKC and with a concentration of 1 μg mL^{-1} for each of the six steroidal standards; an enhancement of signals was observed with a sample injection time of 60 s as compared to the signals of MEKC with a sample concentration of 100 μg mL^{-1} and a sample injection time 3 s. However, as the sample injection time was prolonged to 180 s for the sweeping-MEKC, the separation for α-estradiol, estrone, and testosterone became worse. In order to obtain an ideal separation of these steroids with a long injection time, the concentration of SDS was increased to augment the theoretical plates of the pseudostationary phase. Meanwhile, the concentration of β-CD could also be increased to improve the separation for the two pairs of epimer. In this way, the injection time of the sample could be extended considerably. However, for a good separation among α-estradiol, estrone, and testosterone, the sample injection time seems limited to 600 s with the use of 80 mM SDS and 14 mM β-CD in the running buffer. The injection time longer than 600 s has been tested for a corresponding increase of SDS and β-CD concentration, however, the endeavor for improving the separation among α-estradiol, estrone, and testosterone was in vain. In addition, as the concentration of SDS was increased, the migration time of the six steroids was also increased. Probably, a longer injection time more than 600 s could be obtained by increasing the column length. However, our interest in the present case was not focused on that. The electropherogram for the separation of the six steroids by the on-line sweeping-MEKC with an injection time of 600 s was shown in Fig. 3. It is clear to see that the six steroids are all baseline separated. Note that the baseline separation for α-estradiol (peak 2), estrone (peak 3) and testosterone (peak 4) was shown in the enlarged electropherogram and inlaid in the upper left of Fig. 3. Fig. 3 also indicated that the analysis time was 55 min which is longer than the analysis time of MEKC (40 min). Therefore, the benefit of using a long column length to increase the injection time, thus, to give a more sensitive detection, is partially offset by the increase of analysis time.

3.3. Comparison of MEKC and on-line sweeping-MEKC technique

The performance of the MEKC and the on-line sweeping-MEKC related with the external standard calibration curves of the six steroidal hormones was shown in Table 1. For MEKC, the linear concentration range of the standard calibration curve for the six steroidal hormones was 0.0, 8.0, 10.0, 15.0, 20.0, 25.0, 50.0, and 100.0 μg mL^{-1} and all had excellent linearity ($r^2 \geq 0.9920$). The theoretical LODs and the LOQs of the six steroidal hormones obtained from the method described in the experimental section were 2.2–3.0 μg mL^{-1} and 7.3–9.9 μg mL^{-1}, respectively. Since the detection limit of MEKC tested for the six steroids was around 7.5 μg mL^{-1} which indicates the inclusion of 0.0 μg mL^{-1} in the standard calibration curve is not suitable, for on-line sweeping-MEKC, the linear concentration range of the standard calibration curve for the six steroidal hormones was 0.030–1.0 μg mL^{-1} for β-estradiol, 0.025–1.0 μg mL^{-1} for α-estradiol, estrone, and testosterone, 0.05–1.0 μg mL^{-1} for androstenedione, and 0.075–1.0 μg mL^{-1} for epitestosterone and their linearity (r^2) were from 0.9785 to 0.9941. The corresponding theoretical LODs and LOQs for the six steroidal hormones obtained from the six standard calibration curves were in the range of 0.010–0.073 μg mL^{-1} and 0.033–0.24 μg mL^{-1}, respectively. Therefore, the UV detection sensitivity for MEKC was enhanced about 93, 125, 240, 200, 47, and 32 times for β-estradiol, α-estradiol, estrone, testosterone, androstenedione, and epitestosterone, respectively, with the on-line sweeping-MEKC sample concentration technique.

Table 1
The external standard calibration curve, linearity, LOD, and LOQ of the six steroidal hormones for MEKC and sweeping-MEKC technique.

Steroid	MEKC				Sweeping-MEKC			
	Linear calibration curve	Correlation coefficient (r^2)	LOD (ppm)	LOQ (ppm)	Linear calibration curve	Correlation coefficient (r^2)	LOD (ppm)	LOQ (ppm)
β-Estradiol	$y = 0.1154x + 0.5597$	0.9930	2.6	8.5	$y = 30.55x + 3.14$	0.9833	0.028	0.093
α-Estradiol	$y = 0.1105x + 0.4522$	0.9941	3.0	9.9	$y = 38.97x + 1.82$	0.9924	0.024	0.081
Estrone	$y = 0.1139x + 0.2966$	0.9959	2.4	7.6	$y = 35.44x + 1.87$	0.9941	0.010	0.033
Testosterone	$y = 0.0990x + 0.3724$	0.9934	2.2	7.3	$y = 32.27x + 2.47$	0.9917	0.011	0.037
Androstenedione	$y = 0.0965x + 0.0960$	0.9966	2.5	8.2	$y = 26.22x + 3.18$	0.9785	0.053	0.18
Epitestosterone	$y = 0.0882x + 0.4760$	0.9920	2.3	7.7	$y = 25.60x + 2.47$	0.9813	0.073	0.24

(a) Each of the standard samples were measured five times ($n = 5$).
(b) For MEKC: injection time for 3 s, applied voltage at 25.0 kV, UV detector at 220 nm.
(c) For sweeping-MEKC: injection time for 600 s, applied voltage at 25.0 kV, UV detector at 220 nm.

3.4. Analysis of steroids in yeast mediated stereoselective reduction culture with on-line sweeping-MEKC

The simultaneous separation of the six steroids in the yeast mediated stereoselective reduction culture has been performed by the reversed phase HPLC in our laboratory, but the results were not ideal. Though the MEKC technique can separate the six steroids very easily, the low sensitivity of the UV detector limits the reaction substrates and products must be at high concentration. In order to perform the trace analysis of the six steroidal hormones, the on-line sweeping-MEKC sample concentration technique is mandatory for the trace analysis of the stereoselective reduction cell culture. The electropherograms in Fig. 4 illustrated the results of the five-day successive on-line sweeping-MEKC analysis of the cell cultures of *S. cerevisiae* mediated stereoselective reduction of 1 μg mL^{-1} each of estrone and androstenedione for simultaneous production of estrone and testosterone. Table 2 shows the five-day successive analysis results for estrone, β-estradiol, androstenedione, and testosterone by the on-line sweeping-MEKC. During the five-day reaction period, only β-estradiol and testosterone were found in the cell culture, thus, the stereoselectivity of the yeast mediated reduction was about 100%. The best yield of β-estradiol and testosterone (44.9% and 29.6%, respectively) can be obtained on the second reaction day. The real sample measurement precisions (RSDs) at different days for estrone and androstenedione were ideal (3.2–6.5% and 3.2–6.8%, respectively); however, for β-estradiol and testosterone they were not ideal (7.0–13.7% and 10.7–18.8%, respectively) which is probably due to the lower concentration level.

The analysis accuracies and precisions (RSDs) of the sweeping-MEKC technique for the six steroids were shown in Table 3. The analysis accuracies estimated by the spike experiments at half-day and on day 4 for the six steroids in the reduction cell culture were 95.0–97.1% and 95.1–98.0%, respectively. The analysis accuracies at two concentration levels indicate an accurate on-line sweeping-MEKC sample concentration technique. The spike precisions were ideal that were in the range of 1.8–3.8%. Therefore, the developed on-line sweeping-MEKC sample concentration technique for the analysis of yeast mediated stereoselective steroids reduction is reliable.

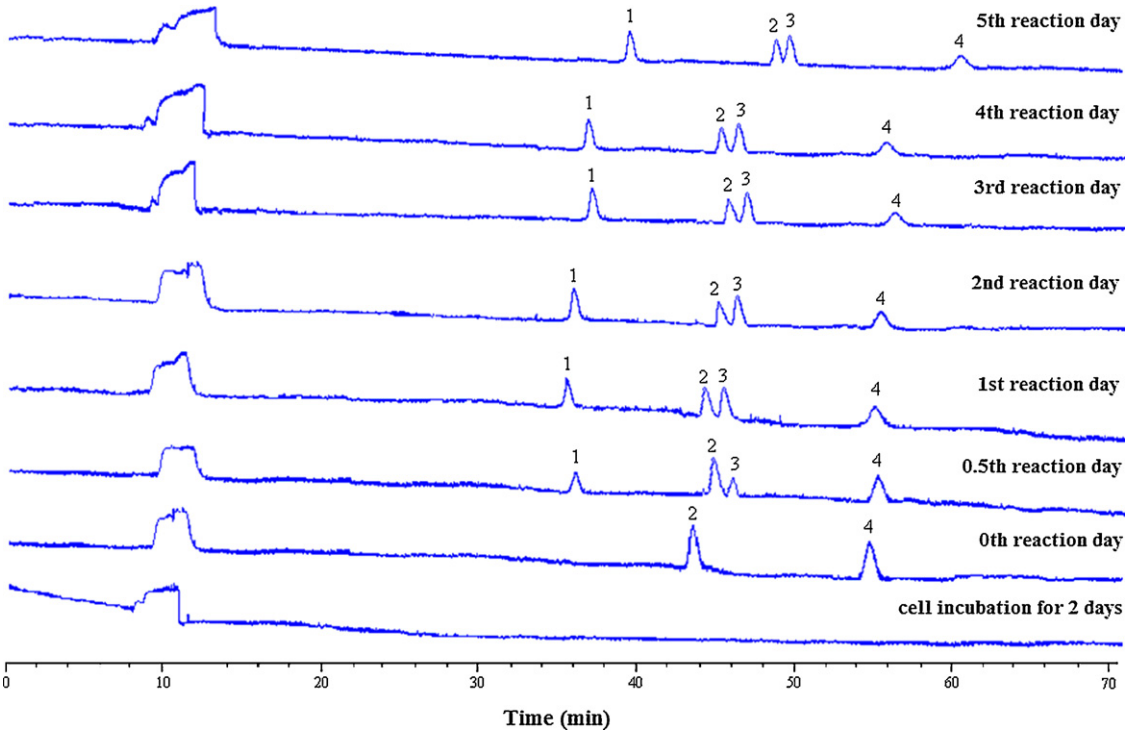

Fig. 4. The analysis of the *S. cerevisiae* mediated simultaneous stereoselective reduction culture of estrone and androstenedione with the on-line sweeping-MEKC sample concentration technique. Substrate concentration: 1 μg mL^{-1} for both estrone and androstenedione. The analysis conditions are the same as Fig. 3. Peak 1: β-estradiol, peak 2: estrone, peak 3: testosterone, and peak 4: androstenedione.

Table 2
The five-day successive analysis results for *S. cerevisiae* mediated simultaneous stereoselective reduction of estrone and androstenedione by on-line sweeping-MEKC sample concentration technique.

Time (day)	Estrone ($\mu g\,mL^{-1}$)	RSD (%)	β-Estradiol ($\mu g\,mL^{-1}$)	RSD (%)	α-Estradiol yield (%)	Androstenedione ($\mu g\,mL^{-1}$)	RSD (%)	Testosterone ($\mu g\,mL^{-1}$)	RSD (%)	Testosterone yield (%)
0	0.95 ± 0.03	3.2	0	–	–	0.94 ± 0.03	3.2	0	–	–
0.5	0.71 ± 0.03	4.2	0.22 ± 0.03	13.7	23.0	0.77 ± 0.03	3.9	0.16 ± 0.03	18.8	16.9
1	0.64 ± 0.03	4.7	0.29 ± 0.03	10.3	30.3	0.60 ± 0.03	5.0	0.27 ± 0.03	11.1	28.5
2	0.50 ± 0.03	6.0	0.43 ± 0.03	7.0	44.9	0.51 ± 0.03	5.9	0.28 ± 0.03	10.7	29.6
3	0.49 ± 0.03	6.1	0.43 ± 0.03	7.0	44.9	0.46 ± 0.03	6.5	0.28 ± 0.03	10.7	29.6
4	0.48 ± 0.03	6.2	0.41 ± 0.03	7.3	42.8	0.46 ± 0.03	6.5	0.27 ± 0.03	11.1	28.5
5	0.46 ± 0.03	6.5	0.39 ± 0.03	7.7	40.8	0.44 ± 0.03	6.8	0.26 ± 0.03	11.5	27.5

The results are obtained within the same day (intra-day) with five repeated measurements ($n = 5$) for each sample.

Table 3
The analysis accuracy and precision of the on-line sweeping-MEKC.

	Spike experiment for the sample of 0.5th reaction day		Spike experiment for the sample of 4th reaction day	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Estrone	97.1	3.5	98.0	2.6
α-Estradiol	95.0	2.4	97.5	2.2
β-Estradiol	95.5	2.7	95.1	1.8
Androstenedione	96.3	3.0	96.1	2.4
Epitestosterone	95.0	2.5	96.7	2.6
Testosterone	95.2	3.8	96.6	3.0

The results are five repeated measurements ($n = 5$) for each sample.

4. Conclusions

An on-line sweeping sample concentration technique coupled MEKC has been developed in this study that can simultaneously separate six estrogens and androgens including two pairs of epimer with a phosphate buffer containing SDS and the stereoisomer selector β-CD. The technique can enhance the sensitivity of UV detector by 32–240 times to that of the MEKC technique. The on-line sweeping-MEKC sample concentration technique was also successfully applied to the study of the *S. cerevisiae* mediated simultaneous stereoselective reduction of estrone and androstenedione at a low concentration that showed a 100% stereoselectivity of the bioreduction toward the production of β-estradiol and testosterone. The analysis of cell culture also demonstrated that the developed on-line sweeping-MEKC sample concentration technique was accurate and precise.

Acknowledgement

The authors gratefully thank the National Science Council for their financial support of this study under grant number NSC 98-2113-M-033-003-MY3.

References

[1] D. Fournier, D. Poirier, M. Mazumdar, S.-X. Lin, Design and synthesis of bisubstrate inhibitors of type 1 17β-hydroxysteroid dehydrogenase: overview and perspectives, Eur. J. Med. Chem. 43 (2008) 2298–2306.
[2] E. Cavalieri, D. Chakravarti, J. Guttenplan, E. Hart, J. Ingle, R. Jankowiak, P. Muti, E. Rogan, J. Russo, R. Santen, T. Sutter, Catechol estrogen quinines as initiators of breast and other hyman cancers: implications for biomarkers of susceptibility and cancer prevention, Biochim. Biophys. Acta 1766 (2006) 63–78.
[3] S.K. Poole, C.F. Poole, Separation of pharmaceutically important estrogens by micellar electrokinetic chromatography, J. Chromatogr. A 749 (1996) 247–255.
[4] E.K. Stürmer, D. Seidlová-Wuttke, S. Sehmisch, T. Rack, J. Wille, K.H. Frosch, W. Wuttke, K.M. Stürmer, Standardized bending and breaking test for the normal and osteoporotic metaphyseal tibias of the rat: effect of estradiol, testosterone, and raloxifene, J. Bone Miner. Res. 21 (2006) 89–96.
[5] T. Piper, U. Mareck, H. Geyer, U. Flenker, M. Thevis, P. Platen, W. Schänzer, Determination of 13C/12C ratios of endogenous urinary steroids: method validation reference population and application to doping control purposes, Rapid Commun. Mass Spectrom. 22 (2008) 2161–2175.
[6] P. Su, X.-X. Zhang, W.-B. Chang, Development and application of a multi-target immunoaffinity column for the selective extraction of natural estrogens from pregnant women's urine samples by capillary electrophoresis, J. Chromatogr. B 816 (2005) 7–14.
[7] R.W. Giese, Measurement of endogenous estrogens: analytical challenges and recent advances, J. Chromatogr. A 1000 (2003) 401–412.
[8] A. Stopforth, B.V. Burger, A.M. Crouch, P. Sandra, The analysis of estrone and 17β-estradiol by stir bar sorptive extraction–thermal desorption–gas chromatography/mass spectrometry: application to urine samples after oral administration of conjugated equine estrogens, J. Chromatogr. B 856 (2007) 156–164.
[9] L. Qiu, W. Liu, M. Huang, L. Zhang, Preparation and application of solid-phase microextraction fiber based on molecularly imprinted polymer for determination of anabolic steroids in complicated samples, J. Chromatogr. A 1217 (2010) 7461–7470.
[10] C. Cheng, H.-R. Tsai, Analysis of steroids in yeast-mediated cell culture by on-line solid-phase extraction coupled high-performance liquid chromatography electrospray-ionization/mass spectrometry and novel continuous postcolumn infusion of internal standard technique, Anal. Chim. Acta 623 (2008) 168–177.
[11] C. Shackleton, Clinical steroid mass spectrometry: a 45-year history culminating in HPLC–MS/MS becoming an essential tool for patient diagnosis, J. Steroid Biochem. Mol. Biol. 121 (2010) 481–490.
[12] D.P. Grove, J.L. Zhou, P.E. Frickers, J.W. Readman, Improved removal of estrogenic and pharmaceutical compounds in sewage effluent by full scale granular activated carbon: impact on receiving river water, J. Hazard. Mater. 185 (2011) 1005–1011.
[13] J.-B. Kim, S. Terabe, On-line sample preconcentration techniques in micellar electrokinetic chromatography, J. Pharm. Biomed. Anal. 30 (2003) 1625–1643.
[14] I. Olędzka, A. Plenis, L. Konieczna, P. Kowalski, T. Bączek, Micellar electrokinetic chromatography for the determination of cortisol in urine samples in view of biomedical studies, Electrophoresis 31 (2010) 2356–2364.
[15] L.K. Amundsen, H. Sirén, Partial filling micellar electrokinetic chromatography analysis of androgens and testosterone derivatives using two sequential pseudostationary phases, J. Chromatogr. A 1131 (2006) 267–274.
[16] K. Németh, G. Tárkányi, E. Varga, T. Imre, R. Mizsel, R. Iványi, J. Visy, J. Szemán, L. Jicsinszky, L. Szente, M. Simonyi, Enantiomeric separation of antimalarial drugs by capillary electrophoresis using neutral and negatively charged cyclodextrins, J. Pharm. Biomed. Anal. 54 (2011) 475–481.
[17] D. Hermawan, W.A.W. Ibrahim, M.M. Sanagi, H.Y. Aboul-Enein, Chiral separation of econazole using micellar electrokinetic chromatography with hydroxypropyl-γ-cyclodextrin, J. Pharm. Biomed. Anal. 53 (2010) 1244–1249.
[18] A.B. Martínez-Girón, A.L. Crego, M.J. González, M.L. Marina, Enantiomeric separation of chiral polycyclic musks by capillary electrophoresis: application to the analysis of cosmetic samples, J. Chromatogr. A 1217 (2010) 1157–1165.
[19] É. Szökő, T. Tábi, Analysis of biological samples by capillary electrophoresis with laser induced fluorescence detection, J. Pharm. Biomed. Anal. 53 (2010) 1180–1192.

[20] M. Silva, Micellar electrokinetic chromatography: methodological and instrumental advances focused on practical aspects, Electrophoresis 30 (2009) 50–64.
[21] H.-X. Chen, X.-X. Zhang, Antibody development to testosterone and its application in capillary electrophoresis-based immunoassay, Electrophoresis 29 (2008) 3406–3413.
[22] C. Desiderio, D.V. Rossetti, F. Iavarone, I. Messana, M. Castagnola, Capillary electrophoresis–mass spectrometry: recent trends in clinical proteomics, J. Pharm. Biomed. Anal. 53 (2010) 1161–1169.
[23] L.K. Amundsen, J.T. Kokkonen, H. Sirén, Comparison of partial filling MEKC analyses of steroids with use of ESI-MS and UV spectrophotometry, J. Sep. Sci. 31 (2008) 803–813.
[24] C.-H. Lin, T. Kaneta, On-line sample concentration techniques in capillary electrophoresis: velocity gradient techniques and sample concentration techniques for biomolecules, Electrophoresis 25 (2004) 4058–4073.
[25] X. Luo, X. Jiang, X. Tu, S. Luo, L. Yan, B. Chen, Determination of malachite green in fish water samples by cloud-point extraction coupled to cation-selective exhaustive injection and sweeping-MEKC, Electrophoresis 31 (2010) 688–694.
[26] M.C. Breadmore, J.R.E. Thabano, M. Dawod, A.A. Kazarian, J.P. Quirino, R.M. Guijt, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2006–2008), Electrophoresis 30 (2009) 230–248.
[27] K.C. Chan, G.M. Muschik, H.J. Issaq, P.K. Siiteri, Separation of estrogens by micellar electrokinetic chromatography, J. Chromatogr. A 690 (1995) 149–154.
[28] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, Pearson Education Ltd., England, 2000.