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Short communication

Separation of structurally related estrogens using isocratic elution pressurized capillary electrochromatography

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Abstract

In this paper, the pressurized capillary electrochromatography (pCEC) with UV detection was utilized for the separation and determination of three structurally related estrogens, such as diethylstilbestrol (DES), hexestrol (HEX) and dienestrol (DE), which were difficult to be separated by capillary electrophoresis (CE) and HPLC due to their similarity in the structure and charge-to-mass ratios. Experiments were carried out in a commercially available pCEC instrument using a capillary column packed with 3 μm octadecyl silica (ODS). Surfactant sodium dodecyl sulfate (SDS) was introduced in the mobile phase to enhance the speed of analysis. The effective factors on the retention time and separation resolution, such as the applied voltage, supplementary pressure, the pH and the concentration of the buffer solution, the concentration of SDS, and the content of acetonitrile in the mobile phase, were evaluated. Based on the investigation, 31% (v/v) acetonitrile and 69% (v/v) of 10 mmol/L phosphate buffer (pH 6.5) containing 1.0 mmol/L SDS at an applied voltage of −12 kV and a supplementary pressure of 1000 psi were found to be the optimal conditions for pCEC to separate the three estrogens. The method also had been applied to the analysis of fish muscle samples spiked with estrogens.

Keywords: Pressurized capillary electrochromatography; Estrogens; Diethylstilbestrol; Hexestrol; Dienestrol

1. Introduction

Capillary electrochromatography (CEC) is a hybrid microcolumn electro-separation technique that couples the high separation efficiency of capillary zone electrophoresis (CZE) with the high selectivity of high performance liquid chromatography (HPLC). Separation can be achieved both by partition and electromigration [1,2]. The unique characteristic of pressurized capillary electro-chromatography (pCEC) is that the electroosmotic flow (EOF) can be combined with pressure, which involves the application of pressure in the electrochromatographic separation by using a HPLC pump [3,4], and changes the near plug-shaped flow. In the case, the pump pressure can be applied as another driving factor to enhance the separation speed and shorten the separation time. This will benefit the separation especially when the electroosmotic flow (EOF) of the column is too low. Another advantage is that the application of pressure in capillary electrochromatography could minimize bubble formation, which will stop the current and destroy the separation [5,6]. CEC separation technique has been proved to be a powerful tool for fast screening and separation, therefore, it has received considerable attention in the analysis of analogs in recent years [7–11].

DES, HEX and DE, a kind of structurally related estrogens which are very similar in structures and effects (see Fig. 1), are widely used in livestock production to promote growth rate and taken as a treatment for estrogen-deficiency disorders in veterinary medicine. However, the research on food toxicology showed that the illegal application of estrogens as repartitioning agents in meat-producing animals [12] became a main hazard to human health, because of their potential carcinogenic properties and other adverse effects. Therefore, fast screening and confirmation of estrogens were important for the investigation of potential use of estrogens in food-safety area. Several methods, namely gas chromatography–mass spectrometry (GC–MS) [13,14], liquid chromatography-iontrap MS [15], voltammetry [16] and on-line SPE-LC-diode array detection [17], had already been reported for the determination of DES either in pharmaceutical formulation, in environment matrices or in biologic

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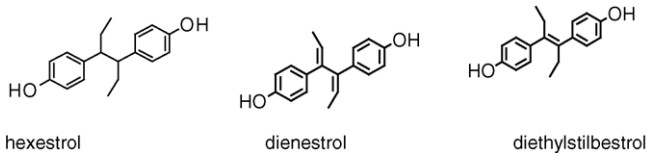

Fig. 1. Chemical structures of the three structurally related estrogens.

matrices. However, the similarity of analytes in the structure and charge-to-mass ratios is the biggest obstacle in separation of other structurally related estrogens. As a result, Capillary electrophoresis, HPLC and other micro-column separation methods have not yet been successfully applied for the simultaneous separation and determination of diethylstilbestrol, hexestrol and dienestrol.

In this paper, a pCEC system with UV detection was developed for the separation and determination of three estrogens. The performance of the pCEC system was evaluated for the purpose of studying and understanding the separation mechanism. The applicability of this residue analysis method has been evaluated with fish muscle samples.

2. Experimental

2.1. Materials

Capillary column 100 μm/375 μm (I.D./O.D.) was obtained from Unimicro Technologies, the total length of the capillary used was 35 cm, of which 20 cm was packed with 3 μm ODS particles. Deionized water was obtained using a Millipore Milli-Q purification system (Milford, MA, USA). DES, HEX and DE were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) and methanol (Chemical Reagent Corporation, Shanghai, China) were of chromatographic grade. Sodium dodecyl sulfate (SDS) (FARCO chemical supplies, Hong Kong), sodium phosphate monobasic, sodium phosphate dibasic dodecahydrate and phosphoric acid (Chemical reagent Plant, Shanghai, China) were of analytical grade.

2.2. Apparatus

pCEC was carried out on a Trisep 2010GV CEC system (Unimicro Technologies, Pleasanton, CA, USA) which comprised a solvent gradient delivery module, a high-voltage power supply (−30 kV and +30 kV), a variable-wavelength UV–vis detector, a micro fluid manipulation module (including a six-port injector) and a data acquisition module. A continuous mobile phase is generated by merging two flows in a mixer and enters six-port injection valve. Samples injected are delivered to the injection valve and introduced [18] in the internal 2 μL sample loop, and then be carried to the four-port split valve by the mobile phase flow. After splitting in a four-port valve, the flow enters a capillary column under constant pressure controlled by a back-pressure regulator. A negative voltage was applied to the outlet of column, and the inlet of column was connected to the split valve and grounded. In this experiment, the isocratic elution system was used and 225 nm was used as the measurement wavelength of the UV–vis detector. The schematics of TriSep-2010GV CEC system is shown in Fig. 2.

2.3. Procedures

Stock solutions (1.0×10^{-2} mol/L) of each estrogens were first prepared in methanol, and diluted by methanol to obtain the desired concentration before use. All samples were protected from light and stored at room temperature. Prior to pCEC analysis, the samples were passed through a 0.45 μm filter.

Extraction of the fish muscle sample was carried out by agitating 2.5 g of fresh fish muscle containing three estrogens additive and 5 ml of methanol in a 10 ml centrifuge tube for 5 min, and ultrasonic extracting for 20 min. Followed by centrifugation for 10 min at 4000 rpm, the upper layer of the mixture was transferred into a test tube and evaporated to dryness in a water bath at 70 °C. The concentrate was final dissolved by 2 ml methanol, and filtered with a 0.45 μm filter before injection.

Mobile phase solutions were prepared as follows: a certain volumes of acetonitrile and phosphate buffer containing defined SDS were mixed. Buffer was prepared from a 0.2 mol/L solution of phosphate salt, pH of phosphate buffer was adjusted using 10% phosphoric acid. Mobile phase solution was degassed in an ultrasonic bath for 15 min before use. A negative voltage was applied to the column outlet and the column inlet was grounded. Pressure was applied to the column inlet during the separation.

Before a pCEC experiments, the column was conditioned on the instrument with the mobile phase for 1 h, applied voltage

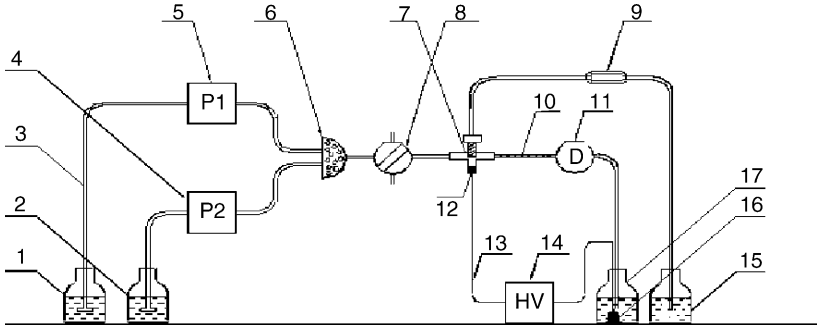

Fig. 2. Schematics of TriSep-2010GV CEC system: (1) mobile phase reservoir one, (2) mobile phase reservoir two, (3) PTFE tube, (4) pump two, (5) pump one, (6) micro-mixer, (7) micro-splitting four-port, (8) six-port, (9) back-pressure regulator, (10) column, (11) detector, (12) grounded electrode, (13) electrical wire, (14) high-voltage power supply, (15) waste one, (16) electrode and (17) waste two.

was first ramped from 0 to −12 kV and then operated at −12 kV. The column was equilibrated for about 30 min after the mobile phase was changed and the temperature of the column was kept at room temperature.

3. Results and discussion

3.1. Optimized conditions for separation of estrogens

The effective factors on the migration behavior of three structurally related estrogens, such as the component of mobile phase, flow rate, applied voltage and back-pressure regulator, have been investigated in detail.

The components of mobile phase, e.g. the concentration of SDS or the content of ACN, is expected to influence various physical parameters (ionic strength, viscosity, dielectric constant) of flow in the pCEC system. In order to examine the effect of organic modifier on the separation of analytes by pCEC, different content of acetonitrile solutions, in the range of 30–34% (v/v), were added to a 10 mmol/L phosphate buffer (pH 6.5) containing 1.0 mmol/L SDS. When the content of acetonitrile was increased, an increase of the electroosmotic flow velocity was observed, which indicated that the separation of the estrogens is faster in mobile phase containing higher content of acetonitrile.

The addition of surfactants into the mobile phases of CEC has been studied by several authors [19–21]. In CEC, SDS has been found to be effective in not only preventing bubble formation by decreasing the surface tension at the solid–liquid interface [22], but also controlling and stabilizing EOF velocity by changing the surface charged density [23]. The EOF velocity was found to increase very quickly at the first stage with a surfactant concentration from 0 to 1.0 mmol/L. A further increase of the SDS concentration brought out a slight increase of the EOF values, which was attributed to the change in zeta potential due to adsorbed SDS molecules. This behavior can be explained more simply by considering the structure of the SDS molecule [24]. Meanwhile, a loss of the resolution between DE and HEX and an increase of the resolution between DES and DE were observed, which could be explained by the change of the capacity factor when the concentration of SDS was increased from 0 to 3.0 mmol/L in the mobile phase. Therefore, 1 mmol/L of SDS was added in the mobile phase as a surfactant-mediator for improving the resolution and analytical speed of analytes.

In our experiments, the pH of buffer solution was measured before mixing with the organic solvent. The dependence of the migration behavior of these three estrogens on the pH was investigated in the range of pH 5.0–7.2. Because the generation of the mobile phase flow depends on the number of dissociated residual silanol groups, high pH values are required for a strong electroosmotic flow and rapid analysis. The migration velocity of all these three estrogens was increased with increasing of buffer pH in the range of pH 5.0–7.2, which owed to the expected increase in silanol group ionisation at higher pH. However, the increase of the buffer pH lead to a slightly loss of resolution. The best results were obtained in a phosphate buffer at pH 6.5, which was used in further measurements.

The change of buffer concentration will modify the double layer on the silica surface and finally change the EOF of the CEC system [25,26]. The common phenomena in CZE is the increase of the buffer concentration will lead to the decrease of the thickness of the electric double layer at the liquid–solid interface and the decrease of the zeta potential [27]. However, a reverse phenomenon was observed in our experiment. The migration velocity of these three estrogens increased slightly with an increase of buffer concentration, which owed to the reduction in solvent viscosity caused by the thermal effect, and therefore increasing the electroosmotic velocity. We also investigated the effect of phosphate buffer concentration on the separation in the range of 6–10 mmol/L, results showed that the resolution slightly increased with the increase of buffer concentration from 6 to 10 mmol/L. However, when the concentration of the buffer solution was over 10 mmol/L, we found that the increase of ionic strength result in a high background current that lead to the high frequency of bubble formation. So the optimization of buffer concentration was 10 mmol/L.

The electrophoretic mobility of all these three estrogens increased rapidly with the increase of applied voltage under constant pressure. Over the range of −6 kV to −12 kV, the optimal resolution among DES, HEX and DE was obtained at −6 kV, but the retention time of 36 min was almost unacceptable. Higher voltages up to −12 kV could be employed to reduce the retention time down to 17 min and still provided a baseline resolution. A further increase in applied voltage decreased the resolution significantly due to the large EOF rate as well as joule heating. Therefore, −12 kV was selected as the optimum voltage to accomplish a good compromise.

In the isocratic elution pCEC experiments, the total flow from the four-port valve was split into two paths, and one went to the packed capillary column whose column pressure was fixed by a back-pressure regulator. In the experiment, dienestrol was chosen to evaluate the effect of flow rate of the pump on the response of estrogens. The result demonstrated that response of dienestrol decreased with the increase of the flow rate from 0.08 to 0.15 ml/min under the same voltage and pressure, which maybe attributed to the loss of the quantity of the analytes in column. However, an increase of the resolution for the three estrogens was observed. Therefore, a constant flow rate (0.1 ml/min) of the pump was used.

The pCEC chromatogram under the optimum conditions for separating three estrogens by using a pCEC-UV system was shown in Fig. 3.

3.2. The linear range, detection limit and precision

A series of standard solutions of the three estrogens with a concentration range of 1.0×10^{-5} to 2.0×10^{-3} mol/L was analyzed under optimum conditions and the calibration parameters were shown in Table 1. The linear ranges of the analytes investigated were between 5.0×10^{-5} and 1.0×10^{-3} mol/L, and the detection limits were lower or near 2.0×10^{-6} mol/L (S/N = 3). Table 1 showed the relative standard deviations (RSD) of peak

Table 1
Precision data ($n = 6$)[a] and linearity results for the determination of the analytes

Analyte	Peak area (mV s)		Linear range (10^{-5} mol/L)	LOD[b] (μmol/L)	Regression equation; Y (mV s) = aX (10^4 mol/L) − b		
	Average	RSD (%)			a	b	r^2
DES	3062	5.01	5–100	4.2	1605.9	123.3	0.9963
DE	6188	6.18	5–100	1.6	3512.9	443.1	0.9981
HEX	4020	5.84	5–100	3.6	1979.9	405.2	0.9926

[a] The concentration of all the three estrogens was 2.0×10^{-4} mol/L.
[b] Detection limit was estimated according to three times the signal-to-noise ratio.

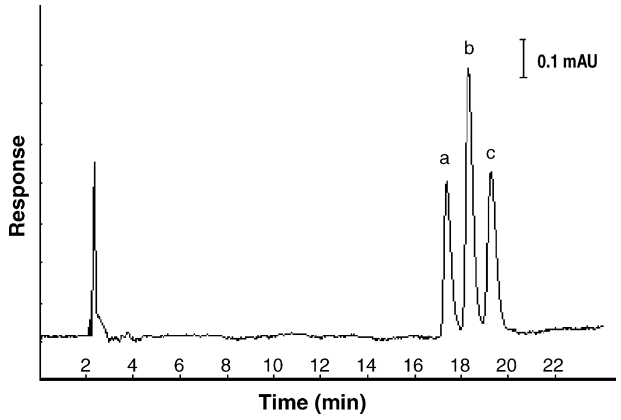

Fig. 3. The pCEC chromatogram under the optimum conditions for separating three estrogens. Experiment conditions: capillary column 100 μm/375 μm (I.D./O.D.), the total length of the capillary used was 35 cm, of which 20 cm was packed with ODS (3 μm), mobile phase: 31% (v/v) acetonitrile, 69% (v/v) of 10 mmol/L phosphate buffer (pH 6.5, containing 1 mmol/L SDS) (31:69) (v/v), applied voltage −12 kV, supplementary pressure 1000 psi, flow rate 0.1 ml/min: (a) DES, (b) DE and (c) HEX.

areas of the analytes when the analysis was repeated six times under the same conditions. All the RSDs were shown as lower than 7.0%, which demonstrated that this method had good reproducibility.

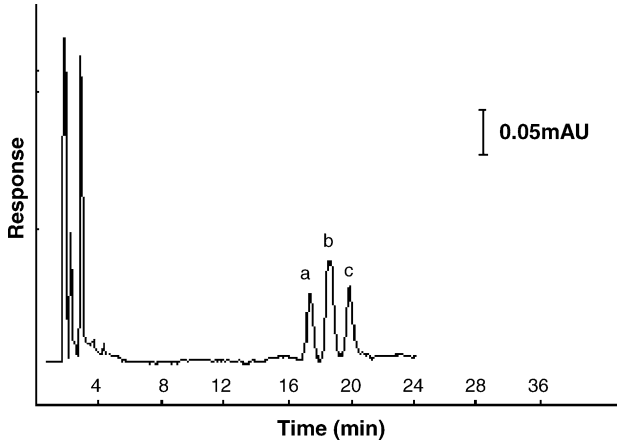

Fig. 4. pCEC Chromatograms of extracts from 1×10^{-4} mol/L DES, DE and HEX spiked fish muscle. Experiment conditions: capillary column 100 μm/375 μm (I.D./O.D.), the total length of the capillary used was 35 cm, of which 20 cm was packed with ODS (3 μm), mobile phase: 31% (v/v) acetonitrile, 69% (v/v) of 10 mmol/L phosphate buffer (pH 6.5, containing 1 mmol/L SDS) (31:69) (v/v), applied voltage −12 kV, supplementary pressure 1000 psi, flow rate 0.1 ml/min: (a) DES, (b) DE and (c) HEX.

Table 2
Recoveries of three estrogens in fish sample analysis ($n = 3$)

Analyte	Added amount (mol/L)	Found amount (mol/L)	Recovery (%)	RSD (%)
DES	1.0×10^{-4}	8.50×10^{-5}	85.0	9.2
	2.0×10^{-4}	1.92×10^{-4}	86.0	6.8
	4.0×10^{-4}	3.56×10^{-4}	89.0	8.0
DE	1.0×10^{-4}	9.00×10^{-5}	90.0	7.8
	2.0×10^{-4}	1.98×10^{-4}	89.0	6.0
	4.0×10^{-4}	3.68×10^{-4}	92.0	6.2
HEX	1.0×10^{-4}	9.30×10^{-5}	93.0	6.4
	2.0×10^{-4}	1.90×10^{-4}	95.0	4.3
	4.0×10^{-4}	3.60×10^{-4}	90.0	5.9

3.3. Applications

Analyses of fish muscle samples spiked with three estrogens, respectively, were carried out to verify the applicability of pCEC by the standard addition method. The chromatogram of fish muscle sample spiked with 1.0×10^{-4} mol/L of three estrogens was shown in Fig. 4. The recovery of the spiked estrogens after the sample preparation procedure was estimated by comparing the estrogens peak area obtained in spiked fish muscle sample with the estrogens peak area obtained with the corresponding standard solution mixture of the estrogens. From this assay (see Table 2), mean values of recoveries of 85.0–89.0% for DES, 89.0–92.0% for DE and 90.0–95.0% for HEX were obtained, respectively, which indicated the reliability of the method for the real sample analysis.

4. Conclusions

A pressurized isocratic elution CEC method was developed for the separation and determination of three structurally related estrogens. All the estrogens were completely separated using a mobile phase consisted of a mixture of acetonitrile and phosphate buffer solution (pH 6.5) containing SDS. The effect of various experiment conditions on the separation of the investigated analytes was studied in order to understand the pCEC separation mechanism. The experiment demonstrated that the supplementary pressure and SDS played an important role in the separation of three estrogens. Furthermore, SDS had been found to be effective in not only preventing bubble formation but also controlling and stabilizing EOF. The supplementary pressure also could be used to overcome the problems of bubble

formation and control the EOF velocity. As thus, the speed of analysis was enhanced dramatically, which would be proved to be significant for the rapid screening in food residue analysis. The applicability of this residue analysis method was also evaluated with fish muscle samples.

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