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Sensitive determination method of estradiol in plasma using high-performance liquid chromatography with electrochemical detection

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Abstract

The improvement in the sensitive determination method of estradiol using HPLC with electrochemical detection is described. The improvement was due to the optimization of the potential applied to the electrode of the analytical cell and employment of a guard cell. The detection conditions were optimized from the electrochemical properties of estradiol in acidic and alkaline eluents. The employment of the guard cell drastically decreased the background noise without any reduction in the response of estradiol, and contributed to improvement in the sensitivity. The optimized method combined with pretreatment by liquid–liquid extraction was applied to the determination of estradiol in rat plasma. The detection limit of 8 pg for the standard solution and 24 pg for the plasma sample, which was about 6–8-fold more sensitive compared to the previous reports, was attained.

Keywords: Estradiol

1. Introduction

The measurement of sex steroid hormones in biomaterials has become important in order to survey the human reproductive system. Various methods for the determination of estrogens using high-performance liquid chromatography (HPLC), which seems to be the most appropriate technique with high sensitivity, selectivity and applicability, have been previously reviewed [1–3], but sufficient sensitivity for the trace level determination to a pg level has not yet been attained. We have been investigating the sensitive determination methods of estradiol and developed a system using peroxyoxalate ester chemiluminescence and attained a detection limit of 4 pg for the estradiol standard solution and 12 pg for a rat plasma sample [4].

Electrochemical detection is one of the most sensitive detection techniques for HPLC analysis, especially for electrochemically highly active compounds such as catecholamines [5,6], and has been increasingly used because of its sensitivity and selectivity. Electrochemical detection can also be applied to the determination of steroid hormones [7–9] and detection limits of 200 pg for serum sample [10] and 50 pg for a standard solution of estradiol [11] were attained.

An important point in developing an electrochemical detection method is to reduce the interference

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derived from any electrochemically active substances in the sample solution or HPLC eluent. It is advantageous if the interfering substance can be electrochemically eliminated. In this study, the electrochemical detector equipped with an analytical cell, which oxidizes and determines estradiol, and a guard cell, which oxidizes the electrochemically active materials in the eluent, are used. It was found that the guard cell was quite effective in eliminating the interference from background substances. The potentials applied to the electrode of the analytical cell and the guard cell were optimized to obtain the highly sensitive detection of estradiol.

The HPLC method under the optimized conditions was applied to the determination of estradiol in a biological sample. Rat plasma was used as a model of the biomaterial. For the pretreatment of the plasma sample, liquid–liquid extraction, which was found to be an effective and simple technique [4], was used. The method developed in this report showed high sensitivity for estradiol both in the standard solution and the biological sample.

2. Experimental

2.1. Materials

17β-Estradiol was obtained from Seikagaku Kogyo (Japan), dissolved in methanol to various concentrations and used as the standard solution. Estradiol in plasma was prepared by spiking a one-tenth volume of estradiol methanol solution in rat plasma (purchased from Rockland, USA).

2.2. Apparatus and operating conditions

The HPLC system was composed of a pump (L6320, Hitachi, Japan), a column oven (L-5030, Hitachi) with a reversed-phase column (Capcell Pak C_8 UG-120, 250×4.6 mm I.D., Shiseido, Japan), an autoinjector (AS-4010, Hitachi), and a recorder-integrator (C-R7A, Shimadzu, Japan). The multi-electrode electrochemical detector (Coulochem II, ESA, USA) equipped with an analytical cell and a guard cell, which was placed in front of the autoinjector in order to oxidize any electrochemical active substances in the eluent, was used. Solutions containing a mixture of 0.1% trifluoroacetic acid (about pH 2)–acetonitrile (50:50), and a mixture of 20 m*M* phosphate buffer (pH 9.0)–acetonitrile (50:50) were used as the eluent at a flow-rate of 0.5 ml/min. The injection volume for the HPLC analysis was 10 μl.

2.3. Optimization of the potential of the electrochemical detector

Estradiol was injected into the HPLC system and the response from the electrochemical detector was measured. The potential applied to the electrode of the analytical cell was optimized by varying the potential from 300 to 800 mV. The obtained HPLC peak area was plotted versus the applied potential. During the optimization, the potential of the guard cell was fixed at zero. After the optimization of the analytical cell, the effect of the guard cell was evaluated by setting the potential of the guard cell at 0 or 1000 mV.

2.4. Pretreatment of the plasma sample

A 100-μl volume of rat plasma spiked with estradiol was diluted with 1.9 ml of water. To this solution, 5 ml of ethyl acetate was added and vigorously shaken. After centrifugation at 3000 rpm for 10 min, 4 ml of the ethyl acetate layer containing estradiol was recovered and dried under a nitrogen stream in a glass tube. The residue was dissolved in 100 μl of methanol, and then 10 μl of this solution was injected into the HPLC system.

3. Results

3.1. Selection of HPLC eluent

Fig. 1 shows the chromatograms of estradiol (10 ng injection) in an acidic eluent and detected under various detection conditions. Estradiol was eluted at the retention time of 11.0 min, and detected by the electrochemical detector. In order to select the appropriate eluent, the electrochemical properties of estradiol in acidic and alkaline eluents were measured, because the sensitivity of the electrochemical detection of estradiol depends on the pH of the eluent [12]. The peak areas versus applied potentials

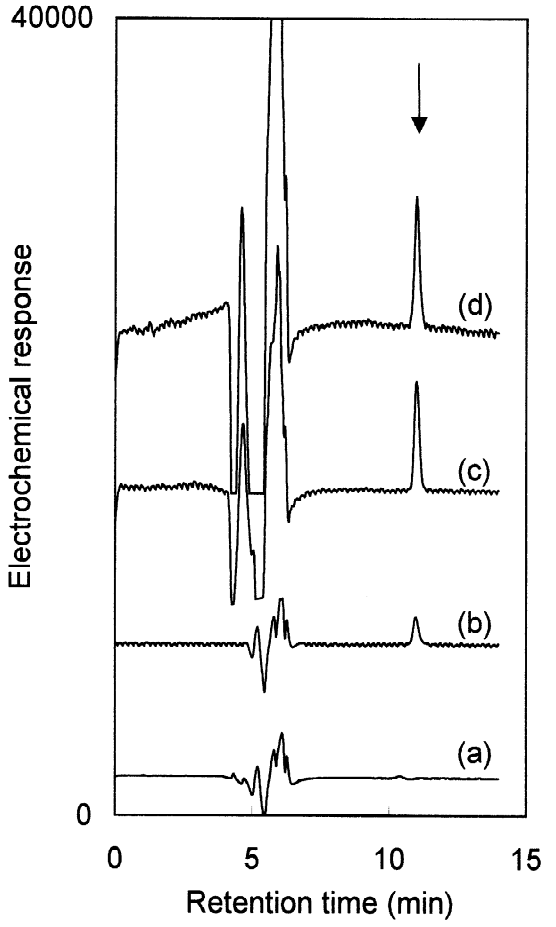

Fig. 1. Chromatograms of estradiol (10 ng injection) detected by electrochemical detection in acidic eluent. The applied potential of the analytical cell was (a) 300 mV; (b) 400 mV; (c) 500 mV; (d) 700 mV. The estradiol peak is indicated by the arrow.

are plotted in Fig. 2. The electrochemical response in the acidic eluent was found to be greater than that in the alkaline eluent, thus, the acidic eluent was used for further studies.

3.2. Optimization of applied potentials of electrochemical detectors

The potential applied to the analytical cell was optimized. As shown in Fig. 1, the chromatogram did not show the estradiol peak when the applied potential was 300 mV (Fig. 1a). It was found that estradiol was oxidized more efficiently as the potential applied to the analytical cell increased from 400 to 700 mV (Fig. 1b–d). The optimum potential applied to the analytical cell was considered to be 700 mV, because the electrochemical response decreased at 800 mV (Fig. 2).

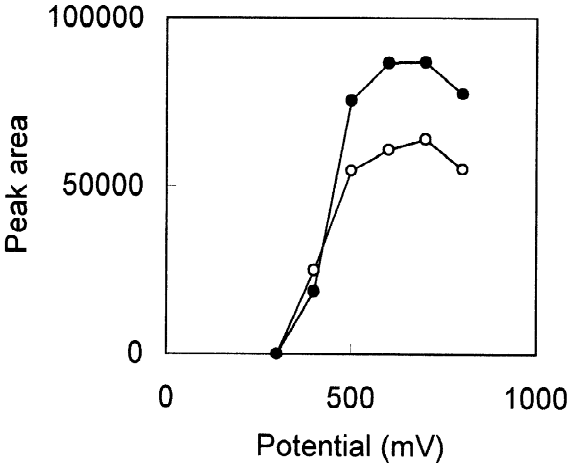

Fig. 2. Electrochemical properties of estradiol in the acidic ([filled circle]) and alkaline ([open circle]) eluents. The horizontal axis indicates the potential applied to the analytical cell.

The background noise also increased according to the increase in the applied potential. Thus, the guard cell was placed in front of the autoinjector in order to suppress the background noise. The potential of the guard cell was set at 1000 mV, higher than the potential of the analytical cell in order to oxidize any electrochemically active substances in the HPLC eluent before introduction into the analytical cell. The effect of the guard cell was ascertained by comparing the chromatograms with or without the guard cell. As shown in Fig. 3, it was demonstrated that the employment of the guard cell drastically decreased the background noise without any reduction in the response of the estradiol peak. The guard cell also contributed to the stabilization of the baseline.

3.3. Determination of the estradiol standard solution

Under the optimized conditions, a calibration curve was established using the estradiol standard solutions. The linearity was good (the correlation coefficient=0.996, $n=5$) in the range between 24

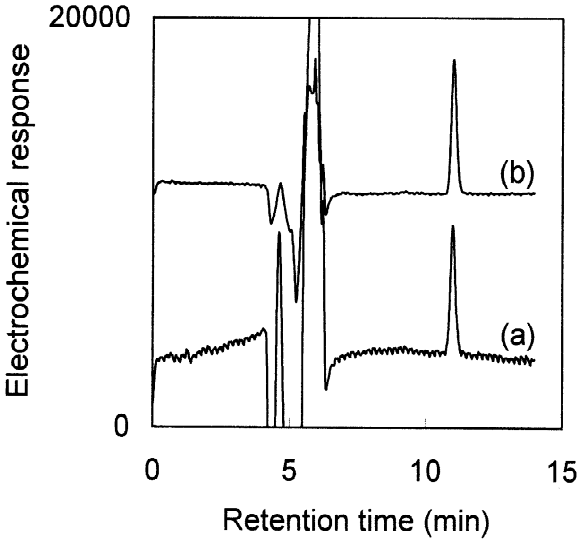

Fig. 3. Chromatograms of estradiol without (a) and with (b) the guard cell.

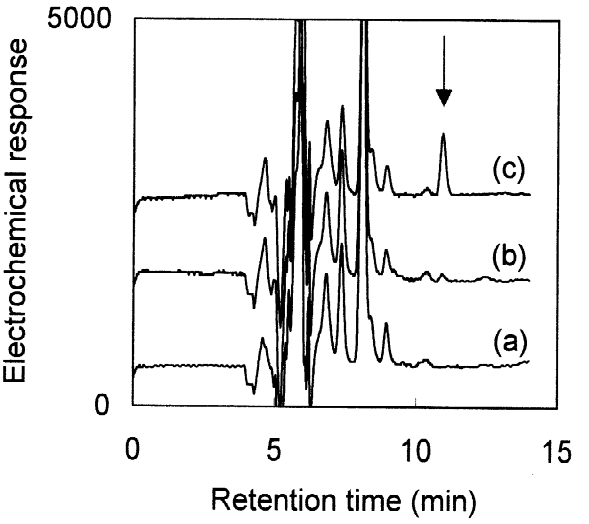

Fig. 5. Chromatograms of estradiol in rat plasma sample detected by electrochemical detection: (a) blank; (b) 80 pg estradiol; (c) 800 pg estradiol. The estradiol peak is indicated by the arrow.

and 2400 pg injections. The chromatograms of the 80 and 800 pg estradiol injections, which correspond to the estradiol standard solutions of 8 and 80 ng/ml, respectively, are shown in Fig. 4. The detection limit for the amount on the column was estimated to be 8 pg at $S/N=3$. The RSD value was 4.2% for the 80 pg injection ($n=3$).

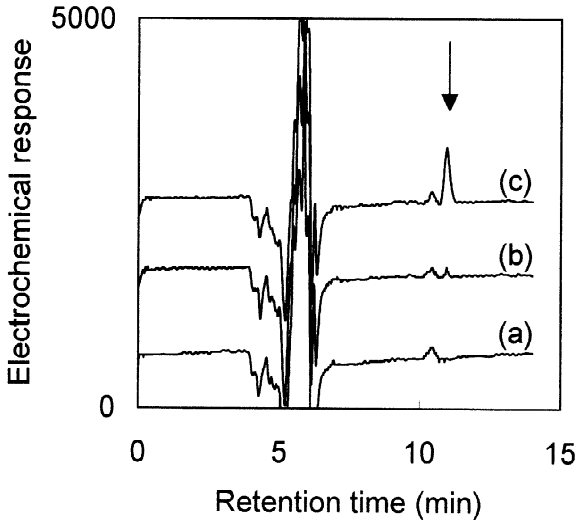

Fig. 4. Chromatograms of the estradiol standard solutions detected by electrochemical detection: (a) blank; (b) 80 pg estradiol; (c) 800 pg estradiol. The estradiol peak is indicated by the arrow.

3.4. Determination of estradiol in plasma sample

The determination method described above was applied to the determination of estradiol in a biological sample. Estradiol in rat plasma was pretreated by liquid–liquid extraction and applied to the HPLC analysis. The chromatograms of the plasma samples with 80 and 800 pg estradiol injections, which correspond to 10 and 100 ng/ml estradiol in the plasma, respectively, are shown in Fig. 5. Although the interference peaks derived from the plasma appeared on the chromatogram, they were distinctively separated from the estradiol peak. The correlation coefficient was 0.999 in the range between the 80 and 8000 pg estradiol injections ($n=5$). The detection limit for estradiol in plasma was estimated to be 24 pg injection. The concentration of estradiol in the rat plasma at the detection limit was 3 ng/ml, when 100 μl of plasma was used for the analysis. The RSD value was 8.3% ($n=3$) and the recovery was 97.6% ($n=3$) for the 240 pg estradiol injection, which corresponded to 30 ng/ml estradiol in the plasma.

4. Discussion

The sensitive determination method for estradiol using HPLC with electrochemical detection was developed as described above, and the detection limit of 8 pg for the standard solution and 24 pg for the plasma sample, which was about 6–8-fold more sensitive compared to the previous reports [10,11], was attained.

Before the optimization, the eluent for the HPLC analysis with electrochemical detection was selected. The efficiency of the electrochemical reaction depends on the pH of the eluent [12]. Thus, we investigated in this study the efficiency of the electrochemical conversion of estradiol in a pH range available for the HPLC analysis, from 2 to 9, using an acidic or alkaline eluent. The acidic eluent was used in this study, because it was found to be advantageous for the sensitive detection of estradiol.

The improvement was due to the optimization of the potential applied to the electrode of the analytical cell and employment of the guard cell. We optimized the potential applied to the analytical cell in order to obtain the efficient oxidation of estradiol. The guard cell contributed to the reduction of the background noise and stabilization of the baseline. With the guard cell, the background noise was drastically decreased by the pre-oxidizing process of the electrochemically active substances in the eluent without any reduction in the response of the estradiol peak (Fig. 3). The effectiveness of the pre-oxidizing process by the guard cell was evaluated as follows. The background noise with or without the pre-oxidizing process was measured by calculating the standard deviation of the entire output signal digitally obtained from the electrochemical detector for one min. The resulting background noise with the pre-oxidizing process was about one-fifth comparing to that without the pre-oxidizing process. Thus, the reduction in the background noise by the pre-oxidizing process was apparently and statistically confirmed.

We applied the determination method described above to the biological sample. Liquid–liquid extraction with ethyl acetate was found to be an effective and simple technique for the pretreatment of the estradiol sample in biological matrices with good recovery and precision, while solid-phase extraction needs conditioning, washing and a recovery process [4]. This treatment extracts relatively hydrophobic materials including estradiol, thus the major interference material such as water-soluble protein remained in the water phase. In addition, no interference peak extracted from the plasma appeared on the chromatogram up to 30 min after the estradiol peak, suggesting that the method with a relatively short analysis time (15 min) can be applicable to the routine analysis.

This study demonstrated that HPLC analysis with electrochemical detection has improved the sensitivity for estradiol without a tedious pretreatment such as derivatization. Also, this study indicated that highly sensitive detection could be achieved by taking advantage of the electrochemical detection with a pre-oxidizing process for reduction of the interference due to coexisting substances.

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