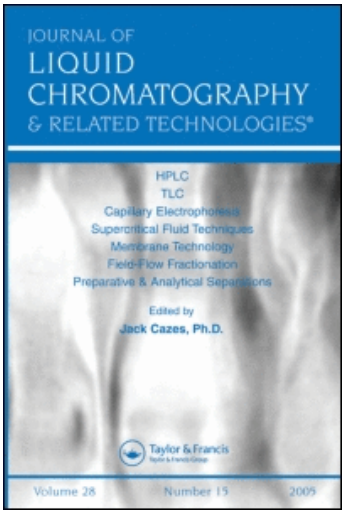

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Amperometric Detection of Estradiol and its Metabolites in Menstrual Women and the Urine of Pregnant Rabbits

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Amperometric Detection of Estradiol and its Metabolites in Menstrual Women and the Urine of Pregnant Rabbits

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Abstract: A liquid chromatography method with electrochemical detection has been developed for the quantitative measurement for the five estrogen derivatives (estrone, estradiol, estriol, 2-methoxyestrone, and ethinylestradiol) in human and rabbit urine. The detection cell consisting of a dual thin-layer glassy carbon electrochemical signal was obtained with a supporting electrolyte containing 40% methanol–25% acetonitrile–35% phosphate buffer (pH 6.0) as the mobile phase. The method was applied for the determination of the four compounds in menstrual women and the urine of pregnant rabbits.

Keywords: Estradiol and its metabolites, Biological urine, Electrochemical detection

INTRODUCTION

Estradiol (E_2) has many biological effects, and some of its metabolites may contribute to the physiological actions of this steroid. Major pathways for the metabolism of E_2 by cytochrome P-450 dependent enzymes in liver microsomes include hydroxylation at the 16α-position to estriol (E_3) and oxidation at the 17β-position to estrone (E_1).[1] Another pathway, 2-hydroxyestrone, is converted into a stable 2-methoxyestrone.[2] Nearly a half century ago, it was recognized that a substantial increase in estrogen excretion accompanied pregnancy. The predominant estrogen was identified as estriol,

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not the usual ovarian estrogens, estradiol or estrone.[3] Main pathways of estradiol metabolism in humans are shown in Figure 1.

Many immunoassays for estriol, with different markers (^{125}I, Tritium and Europium chelates) and detector systems, had been described.[4–6] Derivative UV/VIS spectrophotometry for ethinylestradiol has been reported.[7] Estradiol and estriol cannot be reduced at a dropping mercury electrode because they do not contain the ketonic group or α,β-unsaturated ketonic groups. These compounds must be derivatized and determined by polarography or stripping voltammetry.[8–10] Electrochemcial oxidation of estrogens at a modified carbon paste electrode was not a selective electroanalytical method for the simultaneous determination of some estrogens.[11] Estrogens have been analyzed by HPLC with UV-detection,[12–15] fluorescence,[16–21] electrochemical detection,[22–26] and mass spectrometry.[27–29] HPLC methods following fluorometric pre-column derivatization can improve specificity and sensitivity. However, the derivatization process must be carried out at 60°C for 20 min to form the fluorescent derivatives. In recent years, liquid chromatography/mass spectrometry (LC/MS) has been used for steroid analysis. Although LC/MS techniques have high sensitivity and specificity, and permit rapid sample throughput (i.e., LC/MS methodology, atmospheric pressure chemical ionization (APCI), and electrospray ionization), the mobile phase composition in the analysis of steroids in a biological matrix has not been fully explored, and the instrumentation is expensive.

Most chromatographic papers that have been investigated are of estrogens in serum and tissue. Some investigations deal with the metabolites excreted in the urine using fluorescence detection[14,17,21] and micellar electrokinetic chromatography.[25] There are, however, no reports of the simultaneous determination of estrone, estradiol, estriol, 2-methoxyestrone, and ethinylestradiol, which are present in urine, i.e., the metabolites of 2-methoxyestrone of which there was little reported in the literature. This paper describes an RP-HPLC method with electrochemical detection for the simultaneous determination of estrone, estradiol, estriol, 2-methoxyestrone, and ethinylestradiol after acid hydrolysis of their conjugates in biological fluids.

EXPERIMENTAL

Instrumentation

The HPLC system used consisted of a Hitachi Model L-7110 pump with a Rheodine 7125 injection valve with 20 μL sample loop and coupled with an EG&G PARC 400 electrochemical detector. The flow cell (BAS CC-51) was a confined cross-flow design and the electrodes were as follows: Ag/AgCl/0.1 M KCl reference electrode, stainless steel auxiliary electrode, and dual glassy carbon electrode for the detection of five estrogen derivatives.

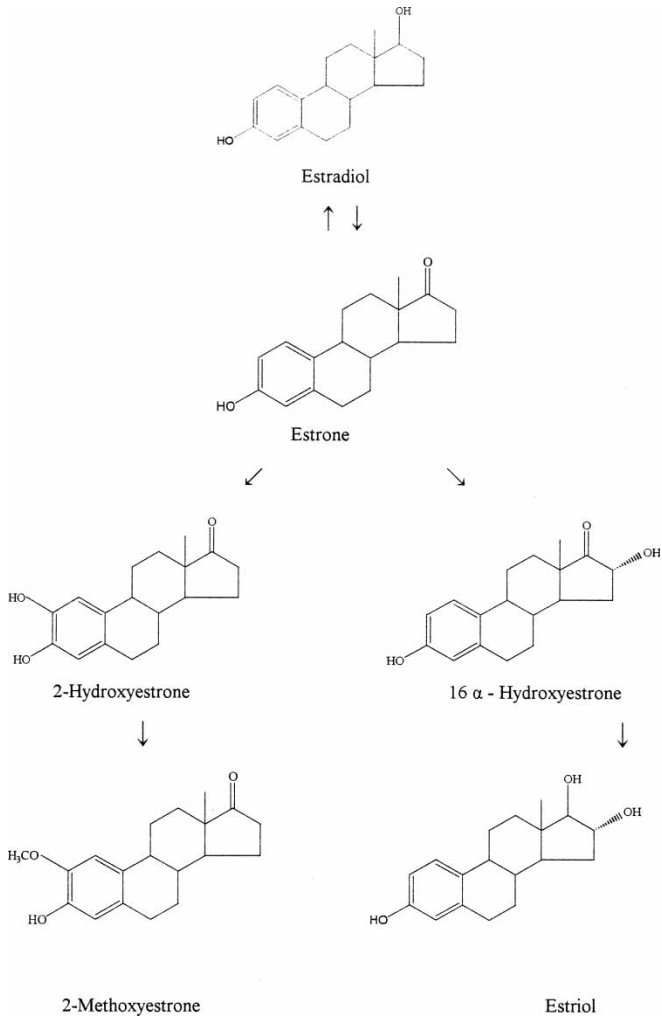

Figure 1. Pathways of estradiol metabolism.

All solvents and analytes were filtrated through a 0.45 μm cellulose acetate and polyvinylidenefluoride syringe (PVDF) membrane filter.

Reagents

The estrogens tested were estrone (E_1), estradiol (E_2), estriol (E_3), and ethinylestradiol from TCI (Tokyo Kasei Co., JP) and 2-methoxyestrone

(2-ME) from Sigma (St. Louis, MO). All other chemicals were of analytical reagent grade.

Human Volunteers

The volunteer population was composed of 5 healthy female subjects (ages 20–24 y) who had not received any estrogens one month prior to participating in the experiment. The urine samples were obtained from the women during the follicular phase of their menstrual cycle.

Rabbits

Female rabbits, weighing 2836–4200 g, were used. The urine was collected for each time period, i.e., 1–31 days during the gestation and stored at −30°C until analyzed.

Extraction of Estradiol and its Metabolites

Sample urine from the rabbits and human volunteers were put into a centrifugal tube containing 4 mL ethanol and centrifuged at 3000 g for 30 min to settle the aggregates, respectively. Rabbit and human supernatant urine (1 mL) were transferred to another reaction vial containing 1 mL 12 M hydrochloric acid and heated with a reflux module at 90–100°C for 15 min. The resulting solution was cooled to room temperature and extracted three times with 10 mL of chloroform. An aliquot of 20 mL of the solution containing 0.1 M sodium hydroxide and 0.1 M sodium bicarbonate was added to the organic phase, washed two times, and then distilled water was used to wash to the organic phase. The organic layer was evaporated at 40°C under dry nitrogen. The dried extract was reconstituted with 0.5 mL of 50% (*v*/*v*) methanol-water and loaded onto a Sep-Pak® C_{18} Waters cartridge, which had been conditioned with 2 mL of methanol and 2 mL water prior to sample loading. An additional 0.5 mL of methanol 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 (eluent discarded), 2.0 mL of 22% acetonitrile–water solution, 1.0 mL of 30% acetonitrile–water solution (estriol), 1.0 mL of 40% acetonitrile–water solution (estradiol), and 1.0 mL of 55% acetonitrile–water solution (estrone, estradiol, estriol, and 2-methoxyestrone). These four fractions were combined and extracted three times with 10 mL of chloroform then dried under nitrogen at 45°C. The dry extract was reconstituted with 1 mL of pure methanol and filtered through 0.45 μm membrane filters before LC analysis.

Determination by Liquid Chromatography

Stock solutions of standards were prepared 1000 μg/mL of estrogens in methanol, respectively. Working standard solutions were prepared from a stock standard solution in methanol in the range 10–1000 ng/mL. A Phenomenex Luna analytical column (particle size 5 μm, 4.6 × 250 mm, purchased from Phenomenex Corporation, USA) eluted methanol–acetonitrile–20 mM phosphate buffer (pH 6.0, 40:25:35, v/v/v) as the mobile phase at 1 mL/min. Detection, after separation on the Phenomenex Luna column, was carried out using an EC detector. The EC detector was operated at +0.9 V. By means of the injection valve, 20 μL of the prepared sample solution and standard solution were chromatographed under the operating conditions described above. Quantitation was based on the peak heights of the sample.

RESULTS AND DISCUSSION

Optimization of LC-ECD Conditions

Estradiol structures are phenolic steroids. The estrogens are electrochemically oxidized on the surface of a glassy carbon electrode. Several operational electrolytes (20 mM potassium dihydrogen phosphate, pH 3.7, 20 mM dipotassium hydrogen phosphate, pH 6.0, 100 mM acetate buffer, pH 4.5; 30 sodium perchlorate, pH 6.9,) were tested to find the optimum one to separate the pathological metabolites of estradiol. Figure 2 shows LC-ECD response (peak height) of estradiol in various supporting electrolytes. The best results were achieved with the operational electrolyte of 20 mM dipotassium hydrogen phosphate, pH 6.0, since the ECD response of the estradiol was found to be much higher than in the other supporting electrolytes. Various methanol/acetate or dipotassium hydrogen phosphate dipotassium ratios (60:40, 70:30, 80:20 v/v) were tried with metabolites of estradiol. Binary methanol-buffer mobile phase failed to resolve estrone and estradiol. The retention times of estrone and estradiol were still the same. Ternary solvent mixtures were tested as mobile phases. We tried to separate the five estrogens using isocratic mobile phases 25:45:30 (v/v/v), 40:20:40 (v/v/v), 45:25:30 (v/v/v), and 40:25:35 (v/v/v), methanol-acetonitrile-phosphate buffer. After various studies of the retention behavior of the estrogens, we achieved baseline separation. Methanol-acetonitrile-phosphate buffer (40:25:35, v/v/v) was found to be the best mobile phase for good resolution and least peak interference by the matrix components. In order to determine the optimum applied potential for electrochemical detection, we constructed hydrodynamic voltammograms (Figure 3) for estrone, estradiol, estriol, ethinylestradiol, and 2-methoxyestrone. For the phenolic group in estrogens, the optimum applied potential seems to be from +0.9 V to +1.0 V.

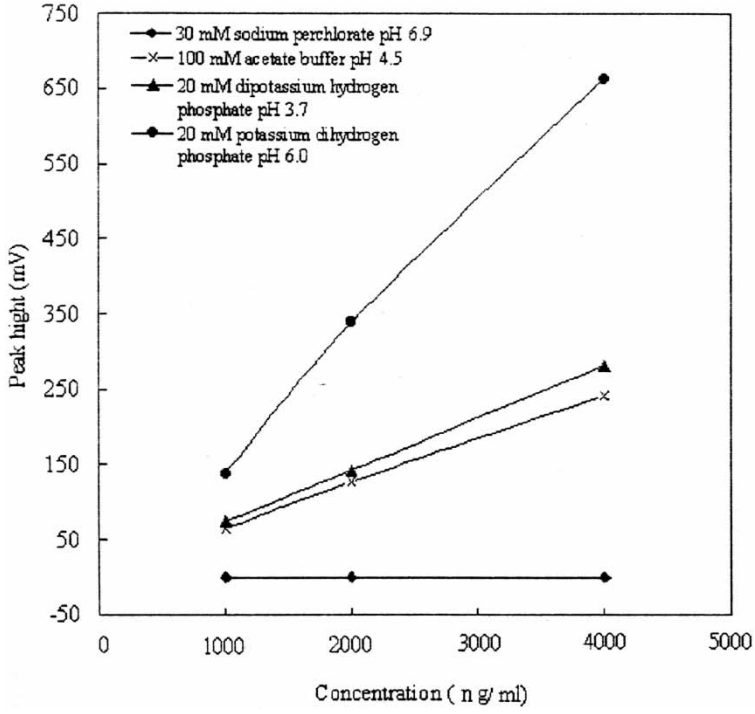

Figure 2. ECD response with estradiol concentration in various supporting electrolytes. Potential, +900 mV *versus* Ag/AgCl.

Linearity and Limit of Detection

The standard curves for all five compounds were determined simultaneously by LC-ECD. Their slopes, intercepts, correlation coefficients, and limits of detection are shown in Table 1. The ECD response showed a linear relationship with the concentration of estrogens over a wide range up to 1000 ng/mL. The limit of detection (LOD) was given by the equation LOD = K So/S, where K was a numerical factor chosen according to the confidence level desired; the standard deviation of the blank measurement (n = 6) and S was the sensitivity of the calibration graph. Here, a value of 3 for K was used, and the LOD are shown in Table 1.

Recovery and Precision

Estrogen mixtures for fortification were prepared by diluting the stock solution with methanol. When real samples are analyzed, some other interferences

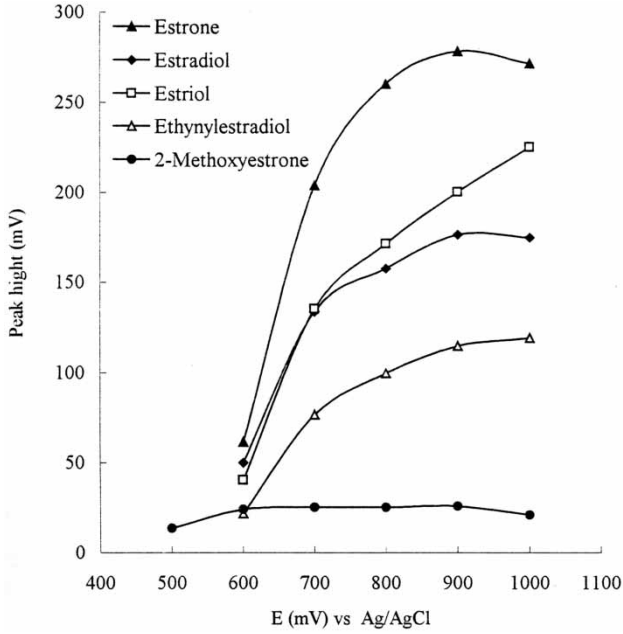

Figure 3. Hydrodynamic voltammograms of estone, estradiol, ethinylestrone, and 2-methoxyestrone 20 ng; conditions: electrode, dual-thin layer glassy carbon electrode; mobile phase, methanol–acetonitrile–water (40:25:35, v/v/v); containing 20 mM phosphate buffer, pH 6.0; flow rate, 1.0 mL/min.

appear in the chromatogram, which need to be separated from the metabolites of interest. We examined interference due to the biological sample matrix by standard recovery studies. A known amount of estrone, estradiol, estriol, and 2-methylestrone standards was spiked into urine samples and into urine

Table 1. The limit of detection (LOD), equations for the linear regression lines and the coefficients of correlation (r) for estrone (E_1), estradiol (E_2), estriol (E_3), 2-methoxyestrone (2 ME) and ethinylestradiol by LC-ECD

	Linear regression line	r	Range of linearity (ng/mL)	LOD (ng/mL)
E_1	$y = 38.1 + 3.34x$	0.9996	50–1000	0.29
E_2	$y = 25.3 + 5.15x$	0.9997	20–1000	0.25
E_3	$y = 25.7 + 8.87x$	0.9994	10–1000	0.46
2 ME	$y = -4.00 + 0.110x$	0.9997	250–1000	2.38
Ethinylestradiol	$y = 2.60 + 0.100x$	0.9999	250–1000	6.98

samples that contained known amounts of endogenous estrogens; extraction was carried out as described above. To calculate percentage recovery, the amount of endogenous estrogens was subtracted from the measured total amount, divided by the added amount, and multiplied by 100. Tables 2 and 3 showed the LC-ECD traces obtained for volunteer and rabbit samples spiked with estrone, estradiol, estriol, ethinylestradiol, and 2-methoxyestrone, respectively; excellent recoveries and precision were observed (recoveries ranging from 81 $\pm$ 3.8% to 101 $\pm$ 4.0%).

Interferences

The chromatogram for the separation of a mixture of estrone, estradiol, estriol, ethinylestradiol, and 2-methoxyestrone is shown in Figure 4. Several peaks which were not identified appear regularly in all chromatograms and, obviously, are not linked to estrone, estradiol, ethinylestradiol, and 2-methoxyestrone, since no peak for them was observed above a retention time of 10 min. Various compounds, such as ascorbic acid, riboflavin (vitamin B_2), pyridoxine (vitamin B_6), uric acid, urea, 4-acetamindophenol, creatinine, and 7-hydroxycoumarin in urine were examined with respect to their interferences with the determination of estriol. Three more electro-oxidative species with retention times of 2.76 min, 2.90 min, and 3.58 min were found in all chromatograms of urine samples; three of them were identified: pyridoxine (RT 2.76 min), 4-acetamindophenol (RT 2.90 min), and 7-hydroxycoumarin (RT 3.58 min). Although these three peaks exhibit retention times very close to that of estriol, none were identical with any metabolites in question.

Table 2. Precision and accuracy in the determination of estrone (E_1), estradiol (E_2), estriol (E_3) and 2-methoxyestrone (2 ME) in human urine sample by LC-ECD[a]

	Added (ng/mL)	Found (ng/mL)	Recovery (%)
E_1	800	737	92.2 (1.4%)[b]
	200	194	97.2 (2.2%)
E_2	800	746	93.3 (1.3%)
	200	193	96.3 (0.9%)
E_3	1,200	1,008	84.0 (3.6%)
	800	711	88.8 (1.4%)
2 ME	800	707	88.4 (3.4%)
	200	202	101.0 (4.0%)

[a]Number of determinations (n = 5).
[b]R.S.D., relative standard deviation.

Table 3. Precision and accuracy in the determination of estrone (E_1), estradiol (E_2), estriol (E_3) and 2-methoxyestrone (2 ME) in rabbit urine sample by LC-ECD[a]

	Added (ng/mL)	Found (ng/mL)	Recovery (%)
E_1	800	734	91.8 (4.1%)[b]
	200	201	100.5 (4.7%)
E_2	800	699	87.4 (3.9%)
	600	513	85.5 (4.9%)
E_3	800	688	86.0 (4.2%)
	600	540	90.0 (1.9%)
2 ME	400	368	92.1 (3.3%)
	200	162	81.2 (3.8%)

[a]Number of determinations (n = 5).
[b]R.S.D., relative standard deviation.

Application to Menstrual Women's Urine

Figure 5 shows a typical chromatogram of the metabolites of estradiol in urine from a normal non-pregnant volunteer obtained using LC-ECD. As can be seen, estrogens do not interfere with the background peaks. When these traces were compared with those obtained from a pure standard sample, constituents with retention characteristics identical to those of estrone, estradiol, estriol, and 2-methoxyestrone were identified and quantified. As shown in Table 4, we examined the urine of five volunteers during their menstrual

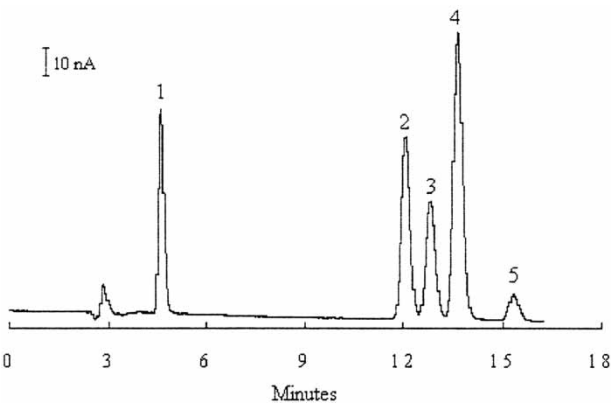

Figure 4. Chromatogram of a mixture of estradiol and its metabolites. The peaks are as follows: (1) estriol; (2) estradiol; (3) ethinylestradiol; (4) estrone; (5) 2-methoxyestrone. Analysis conditions are identical to those listed in Figure 3.

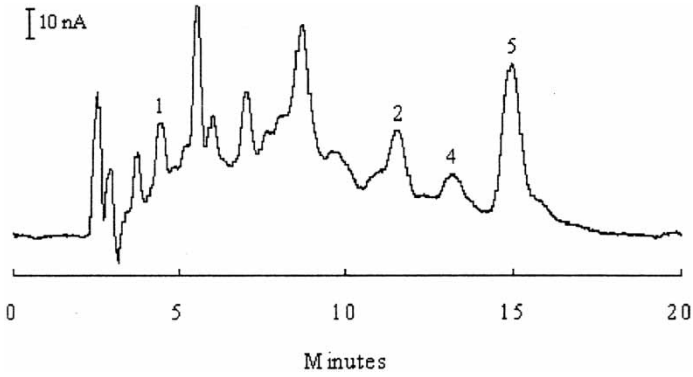

Figure 5. Chromatogram obtained by LC-ECD from urine of a non-pregnant woman (10th day of the menstrual cycle). Peaks: 1 = estriol; 2 = estradiol; 4 = estrone; 5 = 2-methoxyestrone; other peaks are endogenous substance and reagent blank. Analysis conditions are identical to those listed in Figure 3.

periods. The concentration values for the estrone and estradiol in the sample were in agreement with the published (100–200 μg/day).[3] The major metabolite determined by this approach is 2-methoxyestrone and the minor metabolite is estriol. We found that the metabolites of the estradiol were excreted in the urine the 10th day in the menstrual cycle, the average amount being 185 and 335 ng/mL per person for estriol and 2-methoxyestrone, respectively.

Application to Pregnancy Rabbit Urine

The proposed LC-ECD method was applied to the determination of estrone, estradiol, estriol, and 2-methylestrone in rabbit urine. A typical electrochemical

Table 4. Concentrations of estrogens in menstrual women's urine by LC-ECD

	Concentration (μg/day, n = 6)[a]			
Volunteers	E_1	E_2	E_3	2 ME
1	91.0 (5.3%)[b]	110.4 (14.0%)	8.4 (2.6%)	87.6 (14%)
2	ND	ND	60.8 (4.0%)	543.6 (1.3%)
3	33.6 (23.0%)	118.7 (22.0%)	67.2 (10.0%)	355.0 (13%)
4	111.3 (26.0%)	151.2 (30.0%)	262.5 (0.7%)	527.8 (8.1%)
5	ND	ND	ND	329.4 (4.1%)

[a]Number of determinations (n = 6).
[b]Relative standard deviation.
ND, not determined.

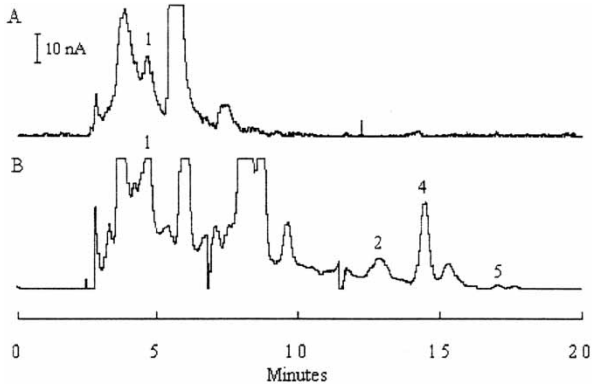

Figure 6. Chromatograms obtained by LC-ECD from before (A) and after (B) urine of a pregnant rabbit (14th day of the menstrual cycle). P eaks: 1 = estriol; 2 = estradiol; 4 = estrone; 5 = 2-methoxyestrone; other peaks are endogenous substance and reagent blank. Analysis conditions are identical to those listed in Figure 3.

Table 5. Concentration of estrogens in pregnant rabbit's urine by LC-ECD

	Concentration (ng/mL, n = 6)[a]			
Days of gestation	E_1	E_2	E_3	2 ME
Before pregnancy	149 (2.3%)[b]	ND	205 (4.7%)	ND
1	61 (4.6%)	ND	54 (3.9%)	62 (3.4%)
2	105 (4.5%)	ND	96 (2.1%)	161 (14.3%)
3	38 (9.9%)	ND	132 (4.4%)	ND
5	172 (0.8%)	ND	185 (24.0%)	ND
7	ND	ND	556 (19.0%)	ND
8	248 (11.0%)	231 (2.7%)	301 (7.7%)	183 (2.8%)
10	ND	ND	572 (6.3%)	85 (2.4%)
12	152 (5.8%)	389 (2.4%)	584 (6.7%)	280 (8.8%)
14	ND	313 (2.7%)	1138 (0.7%)	ND
15	ND	ND	3525 (20.0%)	ND
17	172 (4.6%)	ND	2176 (3.0%)	ND
19	ND	ND	1964 (5.3%)	ND
23	ND	216 (2.8%)	2848 (4.3%)	220 (8.8%)
25	ND	686 (6.1%)	11100 (3.6%)	132 (12.0%)
27	139 (4.8%)	ND	1701 (1.3%)	70 (31.5%)
29	28 (15.0%)	61 (3.5%)	3606 (15.0%)	ND
After pregnancy	174 (2.9%)	ND	272 (5.5%)	ND

[a]Number of determinations (n = 6).
[b]Relative standard deviation.
ND, not determination.

chromatogram for the metabolites, before and after from urine of a pregnant rabbit, was shown Figures 6A and 6B, respectively. Results for the analysis of metabolite derivatives of rabbit urine in the gestation period are shown in Table 5. The urine levels of estrogens vary during the pregnancy and the concentrations of estriol increased with pregnancy and became ten times as high in non-pregnancy. The estradiol and its metabolites were measured during the pregnancy period; the time course of levels in rabbit urine is shown in Figure 7. Concentrations varied from 38 to 248 ng/mL for estrone, 61 to 686 ng/mL for estradiol, 54 to 3606 ng/mL for estriol, and 62 to 280 ng/mL for 2-methylestrone. Peak metabolites occurred during the pregnancy in the 5th day of the gestation collection interval. Estradiol was metabolized to estriol with maximum concentrations of ca. 3606 ng/mL the 29th day after pregnancy. At all the time points tested, estrone, estradiol, estriol, and 2-methylestrone levels were significantly higher in the urine of rabbits for up to 5 days after pregnancy, compared with time zero. Figure 7 illustrates the study of changes in the estrogens during pregnancy. The estriol increased sharply in the later period of gestation, while the levels of the others remained almost unchanged. The curve changes of Figure 7 were

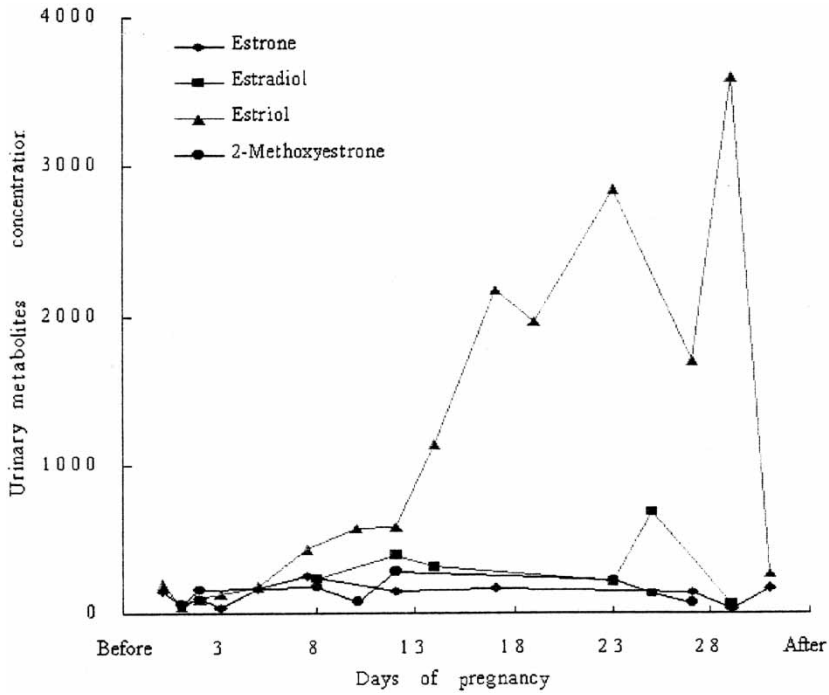

Figure 7. Urinary metabolites of estradiol levels found in a rabbit during gestation.

similar in recent literature in the study of a pregnant woman.[14] Within the 29th day after pregnancy, 92% of estriol derivatives were observed in the urine. The major metabolite determined by this method is estriol and the minor is 2-methoxyestrone.

CONCLUSIONS

The coupling of liquid chromatography with electrochemistry offers a selective and sensitive method for the determination of a wide variety of compounds in body fluids and tissues. With the LC-ECD method described in this study, it is possible to observe that total run times at a flow-rate of 1.0 mL/min were approximately 15 min. Simultaneous determination of the estradiol metabolites should provide a better approach to biotransformations by aryl hydroxylation.

ABBREVIATIONS

Estrone (E_1); estradiol (E_2); estriol (E_3); 2-methoxyestrone (2-ME); HPLC-ECD, high-performance liquid chromatography with electrochemical detection; HPLC-UV, high-performance liquid chromatography with ultraviolet detection.

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