[Chem. Pharm. Bull. 28(8)2512—2514(1980)]

# Electrochemical Detector for High-Performance Liquid Chromatography. III.[1] Determination of Estriol in Human Urine during Pregnancy

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(Received February 8, 1980)

High-performance liquid chromatography combined with electrochemical detection was applied for the determination of estriol in human urine obtained during pregnancy. Estrogens were extracted with ether, chromatographed on a reversed-phase C-18 column with a mobile phase of methanol–water–perchloric acid mixture, and detected electrochemically at 1.1 V *vs.* Ag/AgCl.

The minimum detectable quantity of estriol was 10 ng. Within-run precision was better than 3% for the whole procedure.

**Keywords**——high-performance liquid chromatography; electrochemical detection; determination; estrogen; pregnancy urine

The excretion of estrogens, mainly estriol ($E_3$), into the urine increases steadily during normal pregnancy, and abnormal excretion of estrogens can occur as a result of malfunction of the placenta. Therefore it is important in the clinical control of pregnancy to assay the urinary $E_3$ concentration.

Gas chromatography,[3,4] fluorometry,[5] and high-performance liquid chromatography (HPLC) using an ultraviolet detector[6] have been used for the determination of estrogens. These methods, however, are complicated in operation, and have rather poor sensitivity or selectivity, so it is difficult to determine small quantities of $E_3$, particularly at the early stages of pregnancy. The HPLC method for the determination of $E_3$ as its dansyl derivative[7] has a high background level and the reagent must be prepared freshly each day. The radioimmunoassay method[8] suffers from cross-reactivity.

We have previously described the application of HPLC combined with electrochemical detection (ECD) to the determination of biogenic amines,[9] tocopherols, benzoquinones and naphthoquinones.[1] In this paper, we have extended the method of Shimada *et al.*[10] and developed a HPLC method for the determination of $E_3$ in human urine during pregnancy.

## Experimental

**Apparatus**——The HPLC system used was a Yanagimoto model L-2000, VMD-101. For chromatographic separation, a 25 cm × 4.6 mm I.D. column packed with Nucleosil C-18 (Machery-Nagel., 10 μm) was used. The column temperature and flow rate were maintained at 25 ± 0.1° and 1.0 ml/min, respectively. The applied potential was 1.1 V *vs.* Ag/AgCl reference electrode.

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**Materials**——Estrone ($E_1$), 17$\beta$-estradiol ($E_2$) and $E_3$ were purchased from Sigma Chemical Co., U.S.A. *p*-Methoxyaniline and *n*-valeric anhydride were obtained from Wako Pure Chemical Co., and Tokyo Kasei Co., respectively. *p*-Methoxy-*n*-valeryl-anilide (MVA) was synthesized from *p*-methoxyaniline and *n*-valeric anhydride. All other chemicals were of reagent grade.

**Assay Procedure**——One ml of urine was treated with 0.18 ml of concentrated HCl and the solution was heated at 100° for 30 min. The hydrolysate was shaken with 5 ml of ethereal internal standard solution (containing 300 $\mu$g of MVA in 100 ml of ether) and centrifuged for 2 min at 2000 rpm. The ether layer was washed twice with 1 ml of distilled water and twice with 1 ml of 0.1 M $NaHCO_3$. A 3 ml aliquot of the washed ether layer was evaporated to dryness under reduced pressure and the residue was dissolved in 0.2 ml of ethanol. A 5—20 $\mu$l aliquot of the solution was injected into the HPLC column. The mobile phase was prepared by dissolving 7.0 g of $NaClO_4 \cdot H_2O$ in 1000 ml of methanol–water (6: 4) containing 0.5 ml of $HClO_4$ (70%) solution.

## Results and Discussion

We employed a reversed phase column since it was necessary to use an aqueous organic solvent as a mobile phase which could readily dissolve the supporting electrolyte. The mobile phase thus selected was a mixture of methanol–water–perchloric acid containing $NaClO_4$. The resolution of estrogens was improved by increasing the content of $H_2O$ in the mobile phase up to 45% and the chromatogram was improved by the addition of $HClO_4$. In a mixture of methanol–water–perchloric acid (600: 400: 0.5, v/v) used for the determination of $E_3$, the retention times of $E_1$, $E_2$ and $E_3$ were 15.3, 18.8 and 7.6 min (flow rate; 1.0 ml/min), respectively. The fatty acid derivatives of *p*-methoxyaniline were investigated as internal standards. Fig. 1 shows plots of log $k'$ against the number of carbon atoms in fatty acids as their *p*-methoxyaniline derivatives. On the basis of these data, *p*-methoxy-*n*-valeryl-anilide ($C_5$, MVA) was chosen as an internal standard for $E_3$.

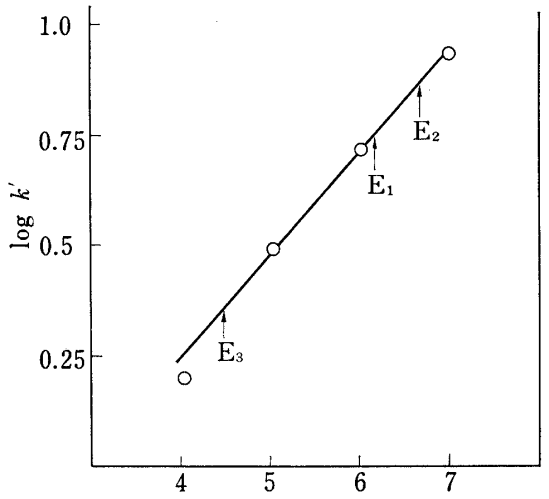


Fig. 1. Plot of log $k'$ *versus* the Number of Carbon Atoms of Fatty Acids as Their *p*-Methoxyaniline Derivatives

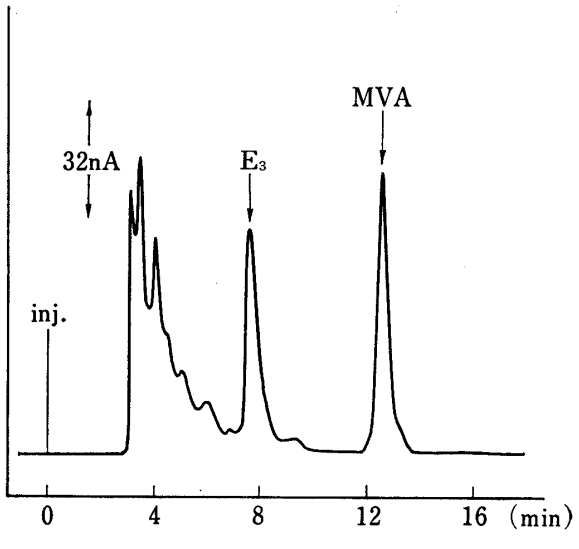


Fig. 2. Chromatogram obtained from a Urine Sample During Pregnancy

Column: Nucleosil C-18 (10 $\mu$m) 25 cm × 4.6 mm I.D.
Mobile phase: methanol: $H_2O$: $HClO_4$ (600: 400: 0.5) containing 7.0 g of $NaClO_4 \cdot H_2O$.
Flow rate: 1.0 ml/min.
Applied potential: 1.1 V *vs.* Ag/AgCl.

$E_3$ in human urine was extracted with ether and determined by HPLC combined with ECD at 1.1 V *vs.* Ag/AgCl. Fig. 2 shows a chromatogram of $E_3$ in human urine. The peak of $E_3$ was identified from the half-wave potential and the UV spectrum measured by the hydrodynamic method during HPLC. The calibration curve of peak height ratio against weight ratio of $E_3$ to MVA was linear from 10 ng to 1000 ng of $E_3$ and the detection limit was

10 ng. The recovery of $E_3$ throughout the whole procedure was 74.4%. The coefficient of peak height variation was 2.7% for 10 μg/ml $E_3$ in human urine (Table I). Table II shows the results obtained from six human urine samples obtained during pregnancy; they are in good agreement with those reported by Mahesh.[11] The excretion of $E_3$ into urine increased with advancing stage of pregnancy, but $E_1$ and $E_2$ could not be detected (the detection limits of $E_1$ and $E_2$ were 10 ng). To investigate unconjugated $E_3$, the urine was directly extracted with ether. However, no free $E_3$ was detected.

Table I. Percent Recovery of Estriol added to Human Urine[a]

| No. | Recovery (%) | |
|---|---|---|
| 1 | 73.1 | $\bar{x}$=74.4% |
| 2 | 74.8 | CV=2.7% |
| 3 | 75.9 | |
| 4 | 75.6 | |
| 5 | 76.1 | |
| 6 | 71.1 | |

a) 10.4 μg of estriol was added to 1 ml of human urine.

Table II. Urinary Estriol in normally Pregnant Women

| Urine sample | Weeks of pregnancy | Estriol(μg/ml) |
|---|---|---|
| 1 | 12.5 | 0.35 |
| 2 | 23.0 | 4.71 |
| 3 | 27.0 | 8.46 |
| 4 | 36.0 | 15.1 |
| 5 | 37.0 | 18.7 |
| 6 | 39.0 | 11.9 |

The present method avoids complicated solvent–solvent extraction and chromatographic procedures, such as paper, thin-layer or column chromatography, reducing the time required for analysis. Furthermore, the sensitivity and specificity are much higher than those of the HPLC method using an ultraviolet detector. Hence, our method should be readily applicable to the monitoring of pregnancy, even at the early stages.

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