Journal of Clinical Laboratory Analysis 4:410–413 (1990)

Measurement of Serum Unconjugated Estriol and Estradiol by High-Performance Liquid Chromatography

Zenjiro Kondo, Tsunehisa Makino and Rihachi Iizuka

Department of Obstetrics and Gynecology, School of Medicine, Keio University, Tokyo, Japan

Measurement of Estriol (E_3) and estradiol (E_2) within 22 min by high-performance liquid chromatography (HPLC) was achieved in this study, and the values were compared with those of a radioimmunoassay (RIA). A totally computerized HPLC method was developed for measuring unconjugated E_3 (u-E_3) and E_2 (u-E_2) in the sera of pregnant women. The serum samples were injected directly into the apparatus and transferred to a pretreatment column where the estrogens were absorbed while hydrophilic components such as proteins and carbohydrates were excluded. The estrogens were then passed through another separation column containing a new type of polymer gel. The mobile phase consisted of an acetonitrile-water mixture, and separation was achieved by means of a reversed-phase procedure. The eluate was monitored for fluorescence. All procedures were monitored and controlled with a built-in microcomputer. Serum samples from 97 normal pregnant women at 20–41 wk gestation were simultaneously assayed by HPLC and RIA. The correlations obtained by HPLC and RIA were as follows: u-E_3, $y = 0.905x - 0.385$, with a coefficient of correlation of $r = 0.912$; for u-E_2, $y = 0.964x + 5.024$, with a coefficient of correlation of $r = 0.841$ (y, RIA value; x, HPLC value). The quick measurement of u-E_3 and u-E_2 by HPLC can be a useful method for evaluating fetoplacental function.

Key words: Estriol, estradiol, high-performance liquid chromatography

INTRODUCTION

Recently, marked advances have been made in prenatal medicine, including early detection of abnormalities of fetal development and estimation of prognosis. Accurate quantitation of estrogens, especially estriol (E_3), is very important in perinatal management since the biosynthetic process of E_3 closely reflects fetoplacental function. Although urinary E_3 determination is a good method for screening because of the simplicity of sampling, its values are influenced by maternal renal function as well as hepatic dysfunction and antibiotic administration.

Blood E_3 values, in particular, unconjugated E_3 (u-E_3) values, are better indicators of fetoplacental function than urinary E_3 values because they show less within-day variation (1), have shorter biological half-lives (2), and are independent of maternal renal function (3,4). Blood E_3 values, however, are generally determined by radioimmunoassay (RIA), which requires not only complex procedures but also much time. Thus, blood E_3 determination has not been practical in clinical medicine.

However, remarkable advances in analytical chemistry have led to the development of various methods for separation of substances. Liquid chromatography is a separation technique originally developed by Twett in 1906. Development of a packing material having a porous surface by Kirkland in 1969 and subsequent studies with this packing material to attain rapid separation have led to the establishment of a modern liquid chromatographic technique that provides rapid flow under pressure: high-performance liquid chromatography (HPLC). Using HPLC, we determined the u-E_3 and unconjugated estradiol (u-E_2) levels in as little as 22 min and evaluated their correlation with the results by RIA.

MATERIALS AND METHODS

Subjects

Sera collected from 97 women in normal pregnancy at 20–41 wk gestation who were outpatients at our hospital were used in this study. Before pregnancy, the women had normal menstrual cycles or had been recording their basal body temperature (BBT). The number of gestation weeks was calculated from the last menstruation or BBT data, respectively.

Received March 23, 1990; accepted June 14, 1990.

Address reprint requests to Zenjiro Kondo, M.D., Department of Obstetrics and Gynecology, School of Medicine, Keio University, 35 Shinano-Machi, Shinjuku-Ku, Tokyo 160, Japan.

HPLC

Reversed-phase partition chromatography (Fig. 1) was performed using both pretreatment and separation columns. The columns were packed with acrylic ester copolymers (10–15 μm diameter), which were made by suspension polymerization of two monomers, tetraethylene diacrylate and tetramethylol methane triacrylate. The columns were stainless steel columns, 4 mm in inner diameter and 20 mm long (pretreatment column), and 6 mm in inner diameter and 250 mm long (separation column).

As shown in Fig. 1, 400 μl of serum from pregnant women was mixed with 150 μl of solution containing 16-epiestriol as the internal standard (IS), and the mixture was automatically pumped into the pretreatment column. Solution B (0.05 M phosphate buffer, pH 7.0) was eluted through the column to remove hydrophilic substances such as proteins and conjugated steroids. Subsequently, solution A (acetonitrile/water 65/35, v/v) was eluted through the column to remove hydrophobic substances such as unconjugated steroids. The eluate was then introduced to the separation column; using solution A, u-E_3, IS, and u-E_2 were eluted (in less-hydrophobic order). By measuring the peaks with a fluorometric detector (exitation, 230 nm; fluorescence, 315 nm), the concentrations of u-E_3 and u-E_2 were calculated from the ratio of u-E_3/IS using a built-in computer system. The computer system had an 8-bit CPU (Z-80, running at 2-mHz) and was used for data processing. The system had 48 kbytes of RAM and was backed up by a lithium oxide battery. All analog signals from the fluorometric detector and a pressure sensor were converted to multiplex digital signals through an analog switch. This microcomputer was able to perform all measurements and data processing and print out the results within about 22 min (Fig. 2).

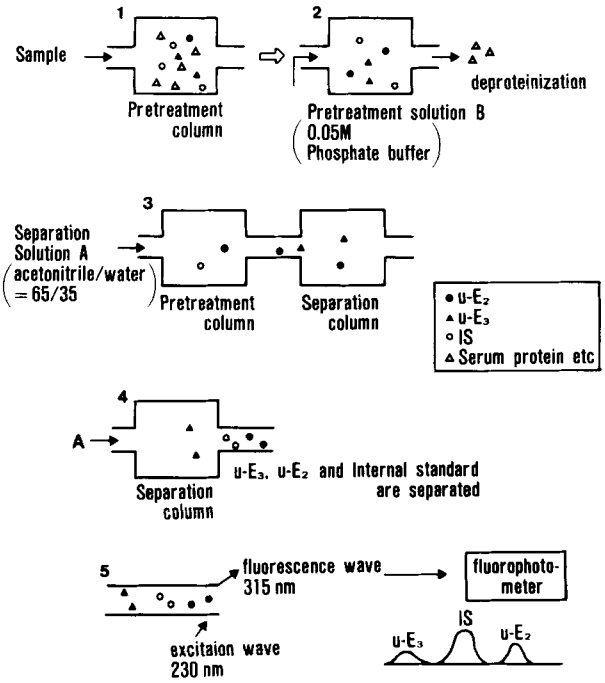

Fig. 1. Separation procedure.

RIA

Serum u-E_3 and u-E_2 were extracted with ether, purified by column chromatography on Sephadex LH-20, and determined by RIA. Rabbit antisera against E_3-6-CMO-BSA and E_2-6-CHO-BSA were used as the antibodies. Separation of free and bound substances was carried out by salting-out with ammonium sulfate. The interassay variance was 15%–20%.

RESULTS

The coefficients of variation (CVs) for the determination with HPLC were as follows: within-day CV for u-E_3, 3.2% (mean u-E_3 = 9.0 ng/ml) to 10.0% (mean u-E_3 = 4.5 ng/ml); within-day CV for u-E_2, 3.6% (mean u-E_2 = 18.8 ng/ml) to 11.0% (mean u-E_2 = 19.3 ng/ml); between-day CVs were 6.3% and 4.9% for u-E_3 and u-E_2, respectively. The recovery rate was about 93%, and the determination sensitivity was 1.0 ng/ml.

The correlations between the results obtained by HPLC (y) and by RIA (x) were as follows. For u-E_3, $y = 0.905x - 0.385$; correlation coefficient, $r = 0.912$ ($n = 97$; Fig. 3). For u-E_2, $y = 0.964x + 5.024$; correlation coefficient, $r = 0.852$ ($n = 97$, Fig. 4).

The serum u-E_3 and u-E_2 concentrations determined by HPLC and RIA in normal pregnant women at various gestations are shown in Figures 5 and 6. As shown in Figure 5, the serum u-E_3 showed a gentle increase until about 34 wk of gestation, but after the 35th wk it rapidly increased in a spike shape to the maximum at the 39th wk; it then showed a decreasing tendency in the 40th and 41st wk. The serum

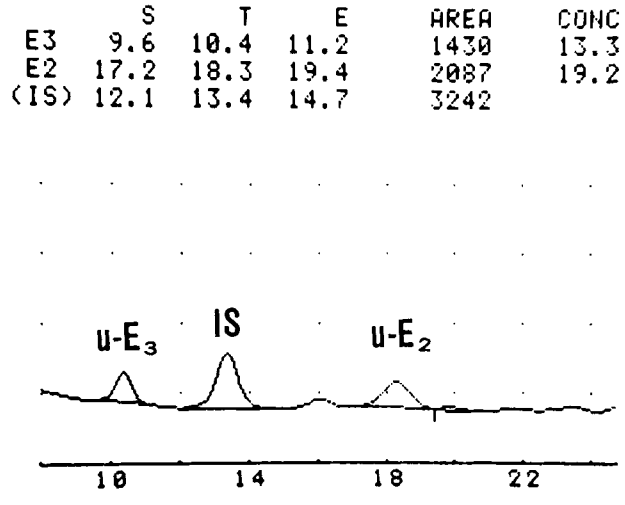

Fig. 2. Serum u-E_3 and u-E_2 peaks on chromatographic chart.

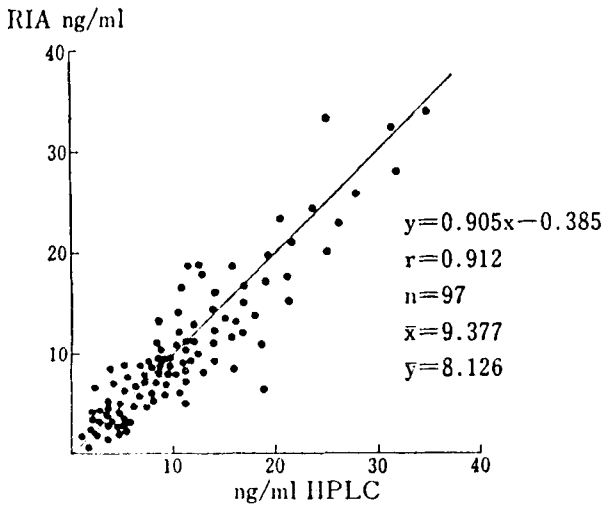

Fig. 3. Correlation between serum u-E_3 concentrations determined by HPLC and RIA for normal pregnant women.

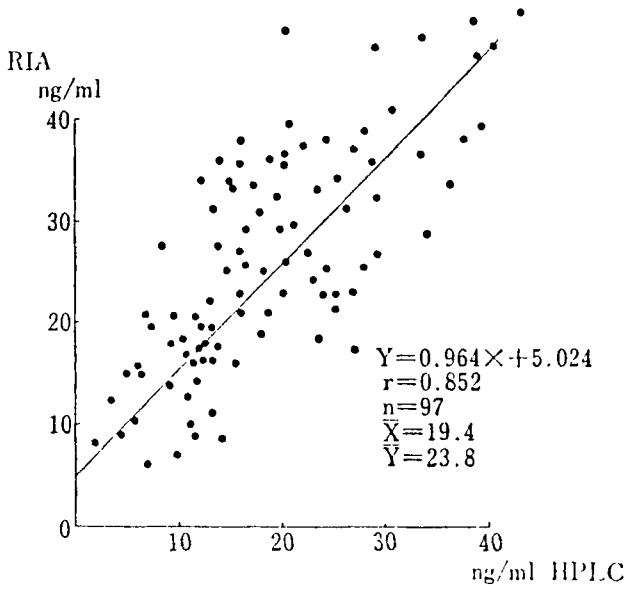

Fig. 4. Correlation between serum u-E_2 concentrations determined by HPLC and RIA for normal pregnant women.

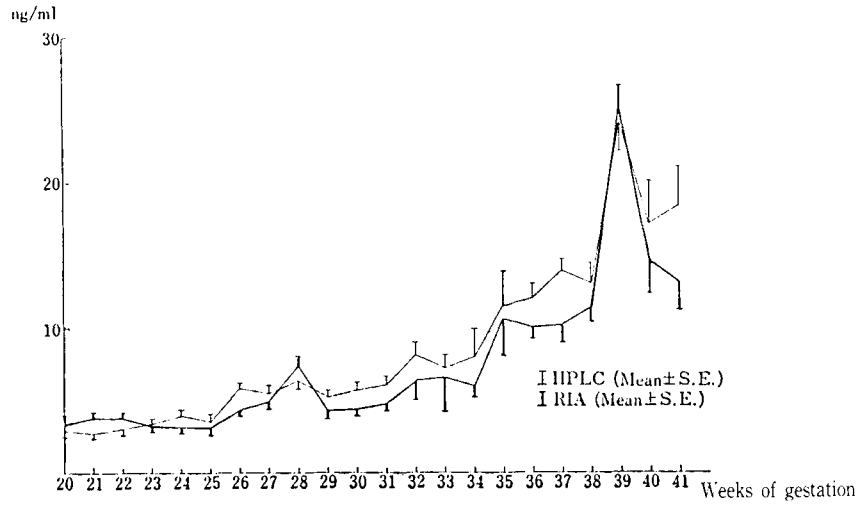

Fig. 5. Serum u-E_3 concentrations determined by HPLC and RIA during gestation.

u-E_2 (Fig. 6) also increased with the weeks of gestation, although in a more linear manner; it decreased in a gentle curve from about the 39th wk.

DISCUSSION

The serum u-E_3 values determined by HPLC showed good correlation with those determined by RIA (correlation coefficient, r = 0.912). In a study using RIA, Tompson and Haven (5) reported that the interassay and intraassay variances were 12.1% and 6.8%, respectively, and the average recovery rate was 85.3%. In our present study using HPLC, the inter- and intraassay variances were 6.3% and 3.2%–10.0%, respectively, and the average recovery rate was about 93.0%. These data suggest that the determination with HPLC is by no means inferior to determination with RIA. Kaplan and Hohnadel (6) determined u-E_3 and u-E_2 by HPLC after extraction of estrogen with ether. The column fillers used in our HPLC were acrylic ester copolymers. As these fillers were made extremely hydrophobic, substances such as u-E_3 and u-E_2 were absorbed in them. Owing to this characteristic, the troublesome procedures of extraction were omitted, and reverse-phase chromatography using a pretreatment column was applied to remove proteins and to concentrate the estro-

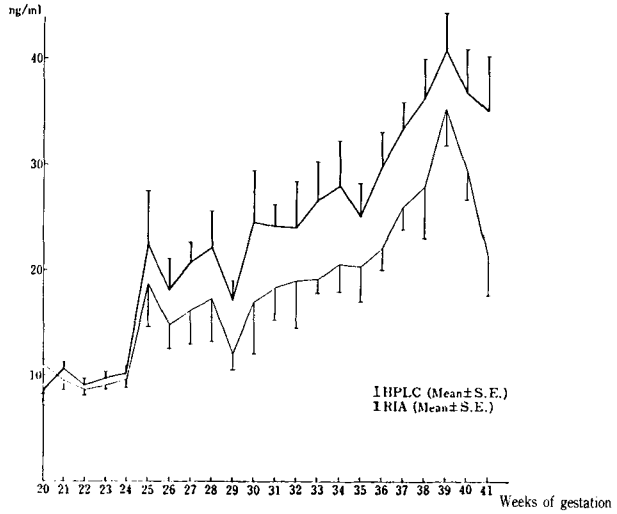

Fig. 6. Serum u-E_2 concentrations determined by HPLC and RIA during gestation.

gens; consequently, u-E_3 values could be determined in as little as 22 min. The u-E_2 concentrations determined by HPLC in this study also showed good correlation with those obtained by RIA (correlation coefficient, r = 0.852). The inter- and intraassay variances were 4.9% and 3.2%–11.0%, respectively.

During pregnancy, 95% of estriol precursors originate from the fetal adrenal glands (7), and estriol is finally biosynthesized in the placenta. Thus, the hormone is produced by interaction between fetal (especially adrenal gland and liver) function and placental function and is therefore very useful in evaluating fetoplacental function.

Assay of urinary E_3 is commonly used as a routine test, since E_3 in blood is finally secreted in an unconjugated form from the placental trophoblast and undergoes conjugation with sulfate or glucoronidation in the maternal liver with a half-life of 15 min. Then 75% of the conjugated form is excreted in the urine; the remaining 25% is excreted in the bile, hydrolyzed by intestinal bacteria, conjugated by glucoronidation by the intestinal mucosa, and finally excreted in the urine. The conjugated E_3 has a half-life of about 2 hr. Taking these aspects of estriol metabolism into consideration, it is believed that unconjugated E_3 is unaffected by renal function, and because its half-life is as short as 15 min, it accurately reflects fetoplacental function at each point. In contrast, urinary E_3, as with conjugated E_3, is liable to be affected by various factors such as maternal hepatic and renal function and enterohepatic circulation (8,9,10). As shown in Figure 5, u-E_3 in women in normal pregnancy increased gradually starting at wk 24–26 of gestation, and it increased rapidly from the 36th wk, reaching a maximum in the 39th; the level tended to decrease thereafter. A marked increase in the fetal adrenal weight and a rapid increase in the fetal cortex weight are known to occur after 7 mo of gestation. Gauthier et al. (10) showed that u-E_3 increased rapidly from the 36th wk of gestation, and parturition began 4 ± 1 wks later; concentrations above 12 ng/ml were considered normal. They also reported that u-E_3 decreased by 12% per week after the 40th wk. Estradiol (E_2) is thought to be inferior to E_3 as an index of fetoplacental function since the precursors of estradiol depend not only on the fetal but also on the maternal adrenal glands, at a ratio of 60:40. Klopper et al. (11), however, suggested that u-E_2 showed slight variation from about the 40th wk of gestation and did not adequately represent fetoplacental function, while some authors, including Tulchinsky et al. (12), found the level to increase gradually. Murakuchi and Tei, although there is no direct correlation between the E_2 level and fetal function, investigated the primary correlation between estrogens (E_2, E_3, and E_4) and fetal factors (weight and length) and reported that E_2 showed the highest correlation with the fetal factors (13,14). In our study, u-E_2 increased gradually in a relatively linear manner, and it could be determined simultaneously with u-E_3 by HPLC.

In summary, HPLC allowed the determination of u-E_3 and u-E_2, which are good indicators of fetal function, without requiring RIA or other troublesome preparative procedures, in as little as 22 min.

REFERENCES

1. Murakuchi K, Ichijo M: Feto-placental function, *Obstet Gynecol Pract* 30:1909–1915, 1981.
2. Klopper AI, Wilson GR, Masson GM: Observation on the variability of plasma estriol. *Obstet Gynecol* 49:459–491, 1977.
3. Kochenour NK: Estrogen assay during pregnancy. *Clin Obstet Gynecol* 25(4):659–672, 1982.
4. Goebelsmann U, Gauthier RJ: The use of estriol as monitoring tool. *Clin Obstet Gynecol* 6(2):223–229, 1979.
5. Tompson J, Haven G: Evaluation of radioimmunoassay for serum unconjugated estriol using commercial reagents. *Am J Clin Pathol* 68:474–480, 1977.
6. Kaplan LA, Hohnadel DC: Unconjugated estriol in serum as measured by liquid chromatography with electrochemical detection, compared with radioimmunoassay. *Clin Chem* 29:1463–1466, 1983.
7. Baulien EE, Dray FD: Conversion of 3H-dehydroisoandrosterone sulfate to 3H-estrogens in normal pregnant women. *J Clin Endocrinol* 23:1298–1301, 1963.
8. Jorge CS, Artal P, Paul RH: Antepartum fetal surveillance in diabetic pregnant patients. *Am J Obstet Gynecol* 141:641–645, 1981.
9. Dubin NH: Comparison of maternal serum estriol and urinary estrogen determinations as indices of fetal health. *Am J Obstet Gynecol* 115:835–841, 1973.
10. Gauthier RJ, Griego BD, Goebelsmann U: Estriol in pregnancy: Unconjugated plasma estriol in prolonged gestation. *Am J Obstet Gynecol* 139:382–389, 1981.
11. Klopper A, Jandial V, Wilson G: Plasma steroid assay in the assessment of fetoplacental function. *J Steroid Biochem* 6:651–656, 1975.
12. Tulchinsky D, Hobel CJ, Yeager E, Marshall JR: Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxy progesterone in human pregnancy. Am J Obstet Gynecol 112:1095–1100, 1972.
13. Murakuchi K, Tei Y: Investigation of changes in serum unconjugated estradiol, estriol and estetrol in pregnancy. *Folia Endocrinol* 57: 974–982, 1981.
14. Murakuchi K: Estriol in pregnant women. *Obstet Gynecol Pract* 36:591–600, 1978.