

Acta Medica Okayama

Volume 39, Issue 3

1985

Article 1

JUNE 1985

Application of Rapid and Simul-taneous Measurement of Sex Steroid Hormones to the Monitoring of Gonadotropin Therapy

Koshi Hayata*

Nobuyoshi Hayashi[†]

Kaoru Sekiba[‡]

*Okayama University,

[†]Okayama University,

[‡]Okayama University,

Application of Rapid and Simul-taneous Measurement of Sex Steroid Hormones to the Monitoring of Gonadotropin Therapy*

Koshi Hayata, Nobuyoshi Hayashi, and Kaoru Sekiba

Abstract

A high performance liquid chromatographic (HPLC) method with both electrochemical detection (ECD) and ultraviolet spectrometric detection (UVD) was developed for the rapid and simultaneous measurement of estradiol (E2), estrone (E1), testosterone (T), 17 alpha-hydroxyprogesterone (17-OHP) and progesterone (P) in serum. These hormones were extracted with diethylether, and chromatographed on an octadecyl silane-silica (ODS) column with an eluent of a phosphate buffer solution - acetonitrile mixture (volume ratio 49:51). Estrogens were detected by ECD at +1.0 V vs. Ag/AgCl, and other hormones by UVD at 242 nm. With this method, the simultaneous determination of sex steroid hormones could be performed within approximately two hours with high precision. The hormones of 34 patients (39 menstrual cycles) undergoing human menopausal gonadotropin (HMG)-human chorionic gonadotropin (HCG) therapy were measured. It was concluded that the switch from HMG to HCG should be performed when the E2 level reaches 400 pg/ml for ovulation and 800 pg/ml for pregnancy. The occurrence of ovarian hyperstimulation syndrome (OHSS) can be predicted when the P level rises above 30 ng/ml on the 7th day after the switch. Moreover, conception may be indicated when the P level does not increase from the 7th to 14th day after the switch. In this way, this method proved to be useful for the monitoring of HMG-HCG therapy.

KEYWORDS: high performance liquid chromatography, electrochemical detection, ultraviolet spectrometric detection, sex steroid hormones, gonadotropin therapy

*PMID: 3927661 [PubMed - indexed for MEDLINE]

Copyright (C) OKAYAMA UNIVERSITY MEDICAL SCHOOL

Acta Med. Okayama 39, (3), 161-170 (1985)

APPLICATION OF RAPID AND SIMULTANEOUS MEASUREMENT OF SEX STEROID HORMONES TO THE MONITORING OF GONADOTROPIN THERAPY

Koshi HAYATA, Nobuyoshi HAYASHI and Kaoru SEKIBA

Department of Obstetrics and Gynecology, Okayama University Medical School, Okayama 700, Japan

(Director : Prof. K. Sekiba)

Received October 1, 1984

Abstract. A high performance liquid chromatographic (HPLC) method with both electrochemical detection (ECD) and ultraviolet spectrometric detection (UVD) was developed for the rapid and simultaneous measurement of estradiol (E2), estrone (E1), testosterone (T), 17 α -hydroxyprogesterone (17-OHP) and progesterone (P) in serum. These hormones were extracted with diethylether, and chromatographed on an octadecyl silane - silica (ODS) column with an eluent of a phosphate buffer solution - acetonitrile mixture (volume ratio 49 : 51). Estrogens were detected by ECD at +1.0V vs. Ag/AgCl, and other hormones by UVD at 242 nm. With this method, the simultaneous determination of sex steroid hormones could be performed within approximately two hours with high precision. The hormones of 34 patients (39 menstrual cycles) undergoing human menopausal gonadotropin (HMG) - human chorionic gonadotropin (HCG) therapy were measured. It was concluded that the switch from HMG to HCG should be performed when the E2 level reaches 400 pg/ml for ovulation and 800 pg/ml for pregnancy. The occurrence of ovarian hyperstimulation syndrome (OHSS) can be predicted when the P level rises above 30 ng/ml on the 7th day after the switch. Moreover, conception may be indicated when the P level does not increase from the 7th to 14th day after the switch. In this way, this method proved to be useful for the monitoring of HMG-HCG therapy.

Key words : high performance liquid chromatography, electrochemical detection, ultraviolet spectrometric detection, sex steroid hormones, gonadotropin therapy.

Due to the recent progress in clinical endocrinology, the measurement of sex steroid hormones in serum has become important. In clinical practice, especially in the monitoring of follicular maturation and placental function, it is essential to measure various sex steroids rapidly. However, it is very difficult with radio-immunoassay.

In this study, a method for the rapid and simultaneous measurement of sex steroid hormones (E2, E1, T, 17-OHP, P) in serum was developed, using HPLC in combination with both ECD and UVD. Additionally, the applicability of the method to the monitoring of gonadotropin therapy was investigated.

MATERIALS AND METHODS

Materials. The standards of E2, E1, T, 17-OHP and P were all the products of Sigma (Missouri). *p*-Methoxy-*n*-caprylic-anilide (MCA), used as an internal standard (IS), was obtained from Eisai (Tokyo). Primary potassium hydrogen phosphate and all the organic solvents (acetonitrile, methanol and *n*-hexane were HPLC grade and diethylether was first grade) were the products of Katayama Chemical Ind. Co., Ltd. (Osaka). In the preparation of samples, a shaker (Yayoi YFS-D) and a centrifugal-evaporator (Yamato RD-21) were used. A high performance liquid chromatograph (Yanaco L-2000), an electrochemical detector (Yanaco VMD-101), an ultraviolet spectrophotometer (Yanaco M-215) and an oven for the electrochemical detector (Yanaco LA-100) were used for the analysis. The column used for separation was an ODS column (Yanapak ODS-T, 4 mm in inner diameter by 250 mm in length).

Preparation of sample. A known amount of MCA was added to 1 ml of serum as an internal standard. Extraction of hormones was performed twice with 4.5 ml each of diethylether with shaking gently. The diethylether layer was washed with 1 ml of 0.1 M sodium bicarbonate solution and 2 ml of water, and then dried at 50°C under a reduced pressure. Two ml each of 70 % methanol and *n*-hexane were added and shaken vigorously, and then the separated layer of 70 % methanol was dried again. The residue was dissolved in 25 μ l of acetonitrile, and 20 μ l of the solution was used for the analysis. The time required for this procedure was 50 min for each sample, and about 2 h for the simultaneous treatment of 10 samples.

Analytical condition. It has been reported that the phosphate buffer solution - acetonitrile system is advantageous as the eluent for the analysis of E2 and E1 by HPLC-ECD using an ODS column (1). Therefore, in this study, the optimum ratio of the mixture was investigated. As shown in Fig. 1, the order of elution was E2, E1, T, 17-OHP, MCA and P, and the separation of the peaks of E2, E1, T and 17-OHP were incomplete. However, as ECD and UVD were entirely specific, it was enough to separate E2 and E1, and T and 17-OHP. The mixture of 0.05 M phosphate buffer solution (pH 3.1) and acetonitrile (volume ratio 49 : 51) was satisfactory as the eluent. The flow rate was maintained at 1.0 ml/min (column pressure 70 kg/cm²),

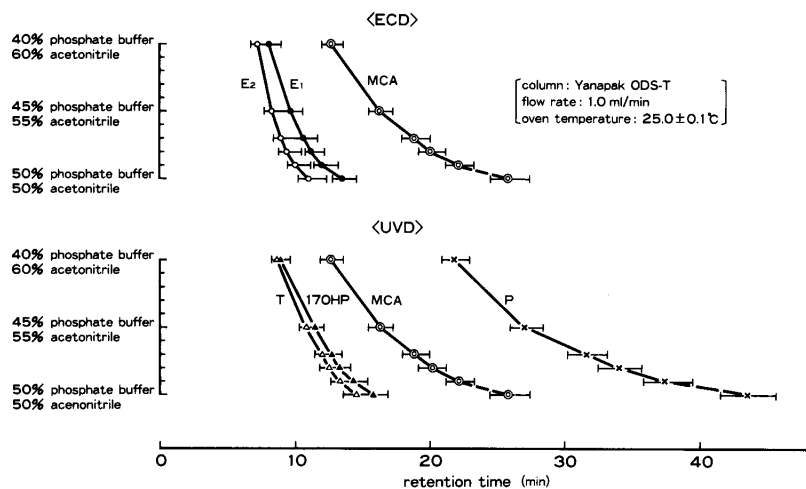


Fig. 1. Composition ratio of eluent

Measurement of Sex Steroid Hormones

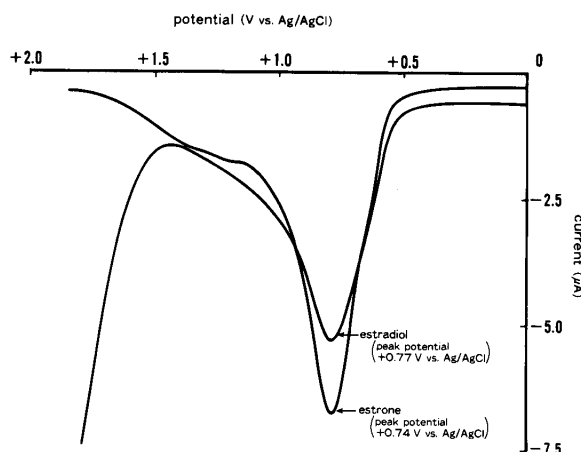


Fig. 2. Electrolytic reaction of estrogen

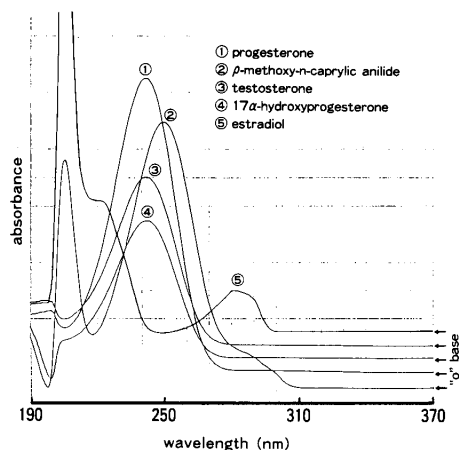


Fig. 3. Ultraviolet absorption spectrum

and the oven temperature at $25.0 \pm 0.1^\circ\text{C}$. Electrochemically active substances can be detected with ECD. Estrogen, which has a phenol body in the A ring of the steroid unit, is oxidized in the ECD electrolytic system, and the current generated is detected. On the other hand, T, 17-OHP and P, having no electrochemical activity, cannot be detected by ECD. The electrolytic reactions of E2 and E1 versus the Ag/AgCl electrode in the eluent were investigated with a differential pulse polarograph (Yanaco P-1000). As shown in Fig. 2, the peak potentials of E2 and E1 were $+0.77$ and $+0.74$ V vs. Ag/AgCl, respectively, and the current limits were reached at $+1.0$ V vs. Ag/AgCl. Therefore, the potential to be applied on ECD was set at $+1.0$ V vs. Ag/AgCl. The ultraviolet absorption spectra of E2, T, 17-OHP, P and MCA in the eluent were investigated with a spectrophotometer (Hitachi 200-20). As shown in Fig. 3, the maximum absorption of T, 17-OHP and P was at the wavelength of 242 nm, and at this wavelength, MCA could be detected, while E2 was scarcely detected.

Clinical study. The hormones of 34 patients (39 menstrual cycles) undergoing HMG-HCG

therapy, where the cervical mucus was used as an index to switch from HMG to HCG, were measured with this method. The classification of OHSS was made by Schenker *et al.* (2), and referred to the degree of ovarian enlargement: more than 12 cm $\times$ 12 cm, severe; below 12 cm $\times$ 12 cm, but more than 5 cm $\times$ 5 cm, moderate; below 5 cm $\times$ 5 cm but ovary palpable, mild; no ovary palpable, no occurrence.

RESULTS

Basic study. Steroid hormone standards and the internal standard were separated and detected satisfactorily by HPLC-ECD, UVD, as shown in Fig. 4. The retention time was E2: 10.2 min, E1: 12.6 min, T: 13.2 min, 17-OHP: 14.2 min, MCA: 22.0 min and P: 38.8 min. The analysis of E2 and E1 was completed in 25 min, *i.e.*, until the formation of the peak of MCA, and the full analysis in 45 min. A stable baseline on the chromatograms was obtained with sensitivities of up to 1 nA full scale in ECD and 0.005 AU full scale in UVD, so that the detection limit with this apparatus (signal/noise ratio = 5) was E2: 32 pg, E1: 38 pg, T: 0.74 ng, 17-OHP: 0.89 ng and P: 1.59 ng. In the cases of actual measurements with serum, the detection limits were about 100 pg/ml for E2 and E1, about 1.5 ng/ml for T and 17-OHP, and about 3.0 ng/ml for P (Table 1). The relationship between the weight of each steroid hormone standard and the height of the peak detected by ECD or UVD showed good linearity, as shown in Fig. 5. Steroid hormone standards (E2: 3.21 ng, E1: 4.35 ng, T: 12.80 ng, 17-OHP: 14.92 ng, P: 32.90 ng) and MCA (22.00 ng) were added to study the recovery and the coefficient of peak height

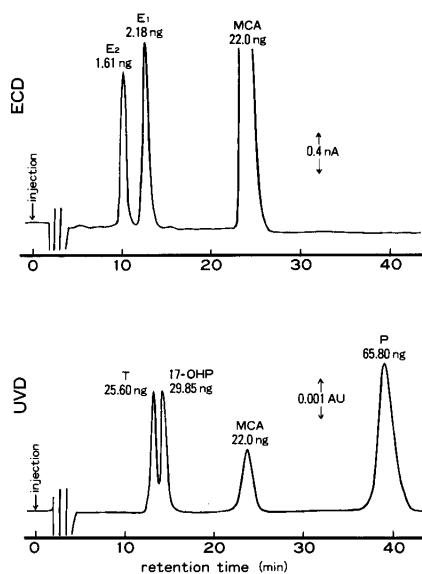


Fig. 4. Chromatogram of standard hormones and internal standard

TABLE 1. DETECTION LIMIT, RECOVERY RATE AND REPRODUCIBILITY

Detection	Pure sample	Pure sample + Serum (n=5)		
	Detection limit	Recovery rate (%)	Coefficient of variation (%)	Detection limit
ECD ^a				
Estradiol	32 pg	80.2	3.5	} about 100 pg/ml
Estrone	38 pg	79.6	3.8	
Internal standard	68 pg	71.8	3.8	
UVD ^b				
Testosterone	0.74 ng	84.1	3.1	} about 1.5 ng/ml
17-OHP ^c	0.89 ng	84.9	3.5	
Progesterone	1.59 ng	61.1	3.5	} about 3.0/ml
Internal standard	1.19 ng	71.3	3.7	

a: Electrochemical detection, b: Ultraviolet spectrometric detection, c: 17 α -Hydroxyprogesterone

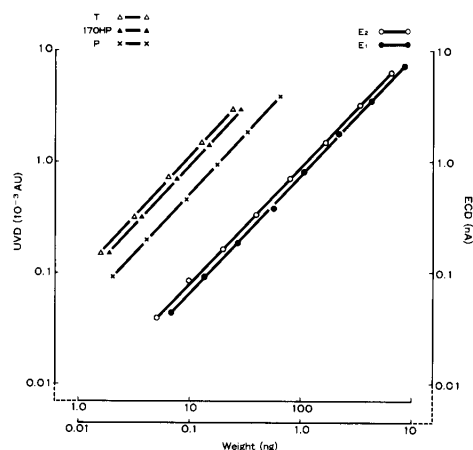


Fig. 5. Calibration line

variation (C.V.). As shown in Table 1, the mean recoveries of E2, E1, T, 17-OHP and MCA were 71.3-84.9 %, and that of P, a slightly lower rate of 61.1 %. C.V. values were 3.1-3.8 %, showing good reproducibility.

Clinical study. The chromatograms (ECD and UVD) of serum extracts obtained at the time of the switch from HMG to HCG, on the 7th day after the switch and at the 9th week of gestation in a woman who had mild OHSS, but successfully became pregnant with twins after HMG-HCG therapy, are shown in Fig. 6. At the time of the switch to HCG, the E2 value was 805 pg/ml, presenting an obvious peak on the ECD chromatogram. The E2 value decreased slightly on the 7th day

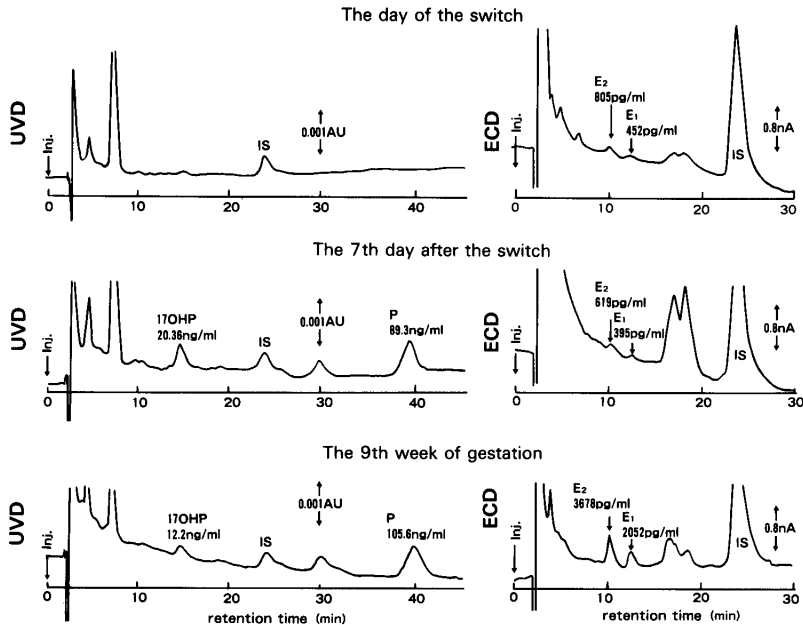


Fig. 6. Clinical application

after the switch, but increased again at the 9th week of gestation. E_1 showed nearly the same pattern as well. Gestagens were detectable on the 7th day after the switch and at the 9th week of gestation, presenting obvious peaks on the UVD chromatograms. T was not detectable at any time.

Next, estrogen values at the time of the switch from HMG to HCG, and gestagen values on the 7th and 14th days after the switch of patients (39 menstrual cycles) undergoing HMG-HCG therapy, were studied as to their relationship to ovulation, pregnancy and OHSS. E_2 at the time of the switch was above 373 pg/ml during the 32 cycles with ovulation, but below 181 pg/ml during the 7 cycles without ovulation, and an obvious difference was noted between them (Fig. 7). In the group with ovulation, E_1 was about 40 % of E_2 , and in the group without ovulation, E_1 was not detected. Concerning the relationship of the E_2 value at the time of the switch in the group with ovulation to OHSS and pregnancy (Fig. 8), mild OHSS appeared even with E_2 (380 pg/ml), and conversely OHSS did not appear in some cases with 1000 pg/ml or more of E_2 . Severe OHSS appeared in 3 cases, showing abnormally high values from 1,300 to 12,821 pg/ml. Seven patients who became pregnant all had values above 805 pg/ml. In addition, no differences were noted between single and twin pregnancies. P on the 7th day after the switch was above 108 ng/ml in cases of severe OHSS, 33-103 ng/ml in moderate and mild cases, and 3.6-28 ng/ml in the group without OHSS (Fig. 9). 17-OHP on the 7th day after the switch was detected in all cases of OHSS, but in 7 of 9 cases without OHSS

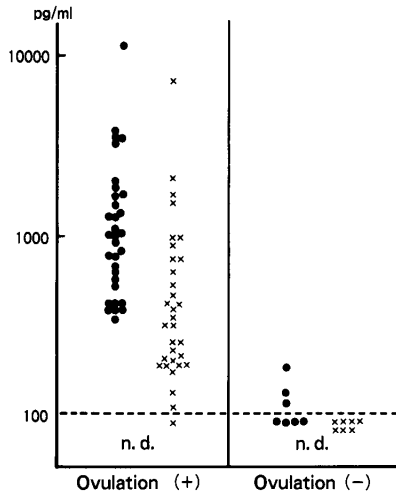


Fig. 7. Estrogen values at the time of the switch from human menopausal gonadotropin (HMG) to human chorionic gonadotropin (HCG). (●) Estradiol, (×) Estrone.

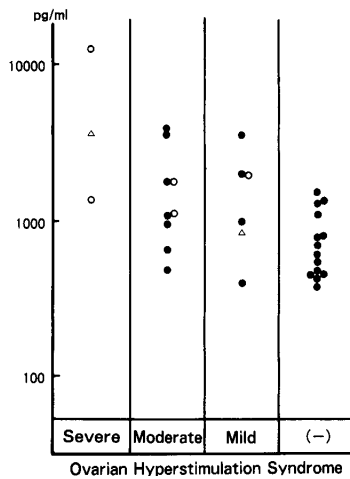


Fig. 8. Relationship of the estradiol value at the time of the switch from human menopausal gonadotropin to human chorionic gonadotropin in the group with ovulation to ovarian hyperstimulation syndrome and pregnancy. (△) Twin pregnancy, (○) Single pregnancy, (●) No pregnancy.

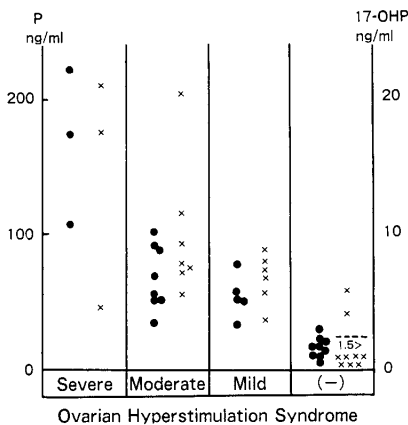


Fig. 9. Relationship of the gestagen values on the 7th day after the switch from human menopausal gonadotropin to human chorionic gonadotropin in the group with ovulation to ovarian hyperstimulation syndrome. (●) Progesterone; (×) 17 α -Hydroxyprogesterone.

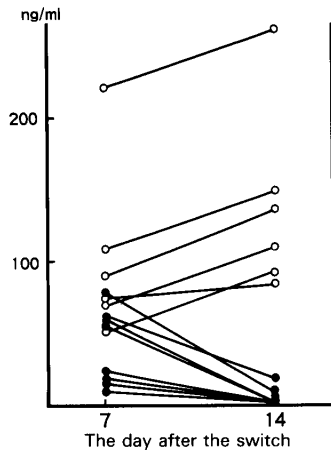


Fig. 10. Relationship of the change of progesterone from the 7th to 14th day after the switch from human menopausal gonadotropin to human chorionic gonadotropin to pregnancy. (○—○) Pregnancy; (●—●) No pregnancy.

it was not detected. In 14 cases where blood was taken on both the 7th and 14th days after the switch, P did not decrease between these two times in patients who became pregnant, but did decrease in those who did not become pregnant (Fig. 10).

DISCUSSION

In HMG-HCG therapy, estrogen in serum is a useful index of when to switch from HMG to HCG. Black *et al.* (3) and Tredway *et al.* (4) used rapid methods of radioimmunoassay capable of measurement within 5 h and reported the usefulness of these methods. Black *et al.* recommended to switch from HMG to HCG when the E2 value rose above 400 pg/ml, and Tredway *et al.* when it was 500-1,000 pg/ml. However, with radioimmunoassay, it is very difficult to obtain the measurement rapidly.

Since Kissinger *et al.* (5) reported a method of measuring catecholamine by HPLC-ECD in 1973, many methods have been developed to measure minute quantities of electrochemically active substances. Recently, a method of measuring serum estrogens by HPLC-ECD was developed (1). With this method, estrone, estradiol, estriol and estetrol above the levels of approximately 500 pg/ml can all be determined simultaneously within 2 h.

However, in clinical practice, especially in the monitoring of follicular maturation, it is desirable to determine E2 and E1 with higher sensitivity. Moreover, in the evaluation of the function of corpus luteum and placenta, it is not sufficient to determine estrogen only; it also is important to determine gestagen and androgen. A method of measuring Δ^4 -3 ketosteroids by HPLC-UVD has already been developed (6). Ikenoya *et al.* (7) reported a method of measuring oxidized and reduced ubiquinone simultaneously by HPLC-ECD, UVD. On the basis of these methods an HPLC-ECD, UVD method for measuring 5 types of sex steroid hormones has been developed in the present study.

It was shown that the present method was excellent in sensitivity, calibration

lines, recoveries and reproducibility, as shown in Figs. 6, 7 and Table 1. Moreover, using MCA as an internal standard, determination was made possible through the correction of the recovery for each sample. This method makes it possible to measure five sex steroids rapidly and to perform profile analysis of these hormones, making it suitable for clinical practice.

The determination of hormones was performed with this method on subjects undergoing HMG-HCG therapy. It was found that the probability of inducing ovulation is high if the switch to HCG is undertaken when the E2 reaches 400 pg/ml. This corresponds to the report of Black *et al.* (3). However, in each of the 7 cases of pregnancy, the switch was undertaken at more than 800 pg/ml, and it proved better to delay the administration of HCG until this level was reached.

The correlation of high estrogen levels in urine with the appearance of OHSS was first reported by Taymor (8). In this study, in only 3 cases of severe OHSS, the switch was made with E2 at more than 1,300 pg/ml, which corresponds somewhat with the reports of Schenker *et al.* (2) and Tredway *et al.* (4). It is desirable to measure E2 frequently in order to prevent an overdose of HMG, and this method appears useful as a rapid assay.

P can be measured with a sensitivity of 3 ng/ml, which is quite satisfactory for observing the function of the corpus luteum after ovulation. In this study, each case that had a P level of over 33 ng/ml on the 7th day after the switch showed OHSS, which was almost identical to the report of Schenker *et al.* (2). Furthermore, the early detection of conception might be possible by knowing the change of P from the 7th to the 14th day after the switch.

17-OHP was detectable at 1.5 ng/ml, and was detected in all cases of OHSS on the 7th day after the switch. However, in 7 of 9 cases which showed no occurrence of OHSS, no peak was noted on the chromatogram. Lehman *et al.* (9) suggested that monitoring levels of 17-OHP during gonadotropin therapy may be useful for predicting the occurrence of OHSS. In this study, it was determined that the occurrence of OHSS could be predicted, depending on whether or not the peak of 17-OHP appeared on the 7th day after the switch.

T can be measured with a sensitivity of approximately 1.5 ng/ml, and in this study it was detected in only 2 cases of polycystic ovary with severe OHSS. The correlation of T to OHSS and multiple pregnancy were reported by Schumert *et al.* (10). Attempts were made to find significant measurements of T concerning these points, but T could not be detected in most cases.

As described above, the HPLC-ECD, UVD system developed in the present study can detect E2, E1, T, 17-OHP and P in serum simultaneously within about 2h with high quantitative accuracy. It was shown that this method was useful in the monitoring of HMG-HCG therapy. This method might also be applicable for the evaluation of general ovarian function and placental function.

Acknowledgment. This work was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture of Japan.

The main points of this article were presented at the 54th Annual Meeting of the Japan Endocrine Society (Tokyo, October 1981), the 34th Annual Meeting of the Japan Society of Obstetrics and Gynecology (Kobe, April 1982) and the 10th World Congress of Gynecology and Obstetrics (San Francisco, October 1982).

REFERENCES

1. Hayashi, N., Hayata, K. and Sekiba, K.: Rapid and simultaneous measurements of estrone, estradiol, estriol and estetrol in serum by high performance liquid chromatography with electrochemical detection. *Acta Med. Okayama* **39**, 143-153, 1985.
2. Schenker, J.G. and Weinstein, D.: Ovarian hyperstimulation syndrome: A current survey. *Fertil. Steril.* **30**, 255-268, 1978.
3. Black, W.P., Coutts, J.R.T., Dodson, K.S. and Rao, L.G.S.: An assessment of urinary and plasma steroid estimations for monitoring treatment of anovulation with gonadotropins. *J. Obstet. Gynecol. Br. Comm.* **81**, 667-675, 1974.
4. Tredway, D.R., Goebelsmann, U., Thorneycroft, I.H. and Mishell, D.R.: Monitoring induction of ovulation with human menopausal gonadotropin by a rapid estrogen radioimmunoassay. *Am. J. Obstet. Gynecol.* **120**, 1035-1040, 1974.
5. Kissinger, P.T., Refshauge, C., Dreiling, R. and Adams, R.N.: An electrochemical detector for liquid chromatography with picogram sensitivity. *Anal. Lett.* **6**, 465-477, 1973.
6. Hiramatsu, U., Hayashi, N., Hayata, K., Akimoto, T. and Sekiba, K.: Daily changes of C₁₉₋₂₁ steroids in neonatal serum by high performance liquid chromatography. *Sanfuchushikaishi* **29**, 81-87, 1980 (in Japanese).
7. Ikenoya, S., Takada, M., Yuzuriha, T., Abe, K. and Katayama, K.: Studies on reduced and oxidized ubiquinones. I. Simultaneous determination of reduced and oxidized ubiquinones in tissues and mitochondria by high performance liquid chromatography. *Chem. Pharm. Bull.* **21**, 158-164, 1981.
8. Taymor, M.L.: Gonadotropin therapy. *JAMA. (J. Am. Med. Assoc.)* **203**, 362-364, 1968.
9. Lehman, F., Neal, C.H. and Bettendorf, G.: Ovarian response during gonadotropin therapy measured by plasma progesterone, 17-hydroxyprogesterone and plasma estrogens. In *Clinical Application of Human Gonadotropins*, ed. G. Bettendorf and V. Insler, George Thieme Verlag, Stuttgart, pp. 113-133, 1970.
10. Schumert, Z., Spitz, I., Diamant, Y., Polishuk, W.Z. and Rabinowitz, D.: Elevation of serum testosterone in ovarian hyperstimulation syndrome. *J. Clin. Endocrinol. Metab.* **40**, 889-892, 1975.