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AUTOMATED DIRECT ASSAY SYSTEM FOR THE MEASUREMENT OF SEX STEROID HORMONES IN SERUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

An automated direct assay system using high-performance liquid chromatography was developed for the simultaneous measurement of estradiol, estrone, progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, testosterone and androstenedione in biological fluids. A comparison between the values measured by this method and by radioimmunoassay revealed good correlation for estradiol ($r = 0.938, p < 0.001$) and progesterone ($r = 0.903, p < 0.001$). Estradiol and estrone could be analysed above the level of 250 pg/ml, and progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, testosterone and androstenedione could be analysed above the level of 5.0–7.5 ng/ml. The method was applied to the clinical appraisal of placental function and maturation of ovarian follicles.

INTRODUCTION

The measurement of sex steroid hormones in the serum has become essential to gynaecological and obstetrical management. In clinical practice, it is important to obtain the results rapidly. High-performance liquid chromatography (HPLC) is superior in simplicity, rapidity and safety to radioimmunoassay (RIA), which is generally used at present. We reported previously the measurement of sex steroids using HPLC: however, the pretreatment was complex [1,10]. Heikkinen et al. [2] reported a simple extraction method for estrogens from urine and plasma using an octadecylsilane bonded-phase disposable packing (9 mm × 10 mm I.D., Sep-Pak C_{18} cartridge). We examined the extraction method for sex steroids from

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serum using the same cartridge and found the method time-consuming. Automated HPLC techniques for estradiol and estriol have recently been reported by Dohji et al. [3]. We modified the methods of Hayata et al. [1] and Dohji et al. [3] and developed an automated system for the measurement of seven sex steroid hormones and applied it to clinical samples.

EXPERIMENTAL

Reagents

The standards of estradiol (E_2), estrone (E_1), progesterone (P), 17α-hydroxyprogesterone (17-OHP), 20α-hydroxyprogesterone (20-OHP), testosterone (T), androstenedione (A-dione), 5α-dihydrotestosterone (DHT) and cortisol (F) were purchased from Sigma (St. Louis, MO, U.S.A.). 4′-Methoxyoctananilide, used as internal standard (I.S.), was obtained from Eisai (Tokyo, Japan). Potassium dihydrogenphosphate and the organic solvents were products of Katayama Chemical Industries (Osaka, Japan).

Samples

Blood samples were taken from subjects between the 18th and 39th week of pregnancy in test-tubes (8.0×1.4 cm I.D.) (Separapid tube, Sekisui Chemical Industries, Osaka, Japan), shaken gently and allowed to stand at room temperature for 10 min. Then they were centrifuged at 1300 *g* for 10 min and the resulting supernatants were stored at −20°C until use.

Samples were analysed both by the present HPLC method and by RIA (CEA-IRE-SORIN kit, France for E_2 and Daiichi kit, Osaka, Japan for P). A pregnant woman's serum in dehydroepiandrosterone sulphate (DHAS) loading test and ovarian follicular fluid were also analysed. In the present method, DHT and F were added to all samples in order to measure both the bound and unbound sex steroid hormones.

Apparatus

A block diagram of the measurement system, which was modified from that of Dohji et al. [3], is shown in Fig. 1. The pretreatment and analytical pumps (Yanaco L-100 and Yanaco L-4000W, respectively, Yanagimoto, Kyoto, Japan) were operated at a flow-rate of 1.0 ml/min. The pretreatment column (30 mm×4 mm I.D.) was a dimethyloctylsilane silica column (Yanapak C_8, Yanagimoto) and the analytical column (250 mm×4.6 mm I.D.) was an octadecylsilane silica column (Yanapak ODS-A, Yanagimoto).

The eluents used in the pretreatment phase were water, methanol and 50 m*M* potassium phosphate buffer (pH 3.1)–acetonitrile (8:2, v/v), as described below. The eluent used in the analytical phase was 50 m*M* potassium phosphate buffer (pH 3.1)–acetonitrile–methanol (10:7:3, v/v). A six-way electromagnetic valve (No. 22) was needed to reverse the flow direction of the pretreatment column during regeneration, and another six-way electromagnetic valve (No. 21) was needed to incorporate this column alternatively into the pretreatment phase and the analytical phase.

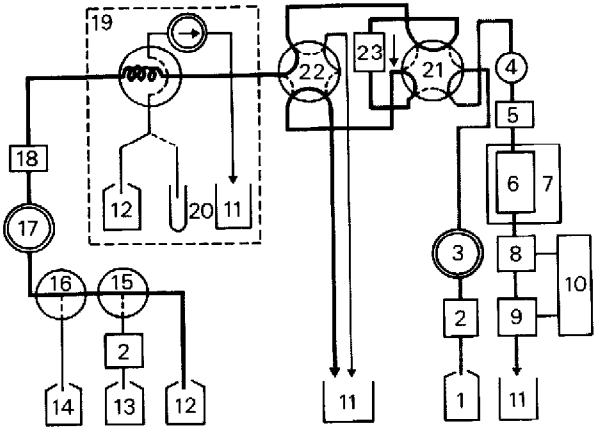

Fig. 1. Block diagram of the system. This shows the first step of the pretreatment.: ———, pretreatment phase; ——, analytical phase. 1=Eluent: 50 m*M* potassium phosphate buffer (pH 3.1)-acetonitrile-methanol (10:7:3, v/v); 2=degasser, 3=pump for analytical phase (flow-rate 1.0 ml/min); 4=manual injector; 5=prefilter; 6=analytical column (Yanapak ODS-A); 7=oven (25.0±0.1°C); 8=UV detector (245 nm); 9=electrochemical detector (applied potential +1.0 V vs. Ag/AgCl); 10=data processor; 11=drain; 12=water; 13=eluent: 50 m*M* potassium phosphate buffer (pH 3.1)-acetonitrile (8:2, v/v); 14=methanol; 15=three-way electromagnetic valve (A); 16=three-way electromagnetic valve (B); 17=pump for pretreatment phase (flow-rate 1.0 ml/min); 18=line filter; 19=autosampler; 20=sample; 21=six-way electromagnetic valve (A); 22=six-way electromagnetic valve (B); 23=pretreatment column (Yanapak C_8).

For the pretreatment phase, an autosampler (Yanaco LS-460) and a degasser (Erma Optical Works, Tokyo, Japan) were used. For the analytical phase, a UV spectrophotometric detector was used at a wavelength of 245 nm (Yanaco M-315), and an electrochemical detector at an applied potential of +1.0 V vs. Ag/AgCl (Yanaco VMD-101A), an oven regulated at 25.0±0.1°C (Yanaco LW-350), a degasser and a chromatogram data processor (Type 7000B, System Instruments, Tokyo, Japan) were also employed. A sequencer (Yanaco AS-100) was used for controlling the system.

Chromatographic procedure

To each 0.2 ml of sample were added 100 ng of 4'-methoxyoctananilide (4 ng/μl in 10% ethanol), 5 μg of DHT (200 ng/μl in 40% ethanol), 5 μg of F (200 ng/μl in 40% ethanol) and 0.25 ml of water.

Sex steroid hormones in a sample were adsorbed onto the pretreatment column, washed with water for 10 min and then washed for 10 min with an eluent of 50 m*M* potassium phosphate buffer (50 m*M* potassium dihydrogenphosphate solution adjusted to pH 3.1 with phosphoric acid) and acetonitrile (8:2, v/v). Because it took more than 20 min for the sex steroids to start eluting from the column with this eluent, these washing steps eliminated protein and other impurities without eluting sex steroids.

Subsequently, the pretreatment column was incorporated into the analytical phase and steroid hormones were eluted with an eluent of 50 m*M* potassium phosphate buffer (pH 3.1)-acetonitrile-methanol (10:7:3, v/v) for 4 min. Sex steroid

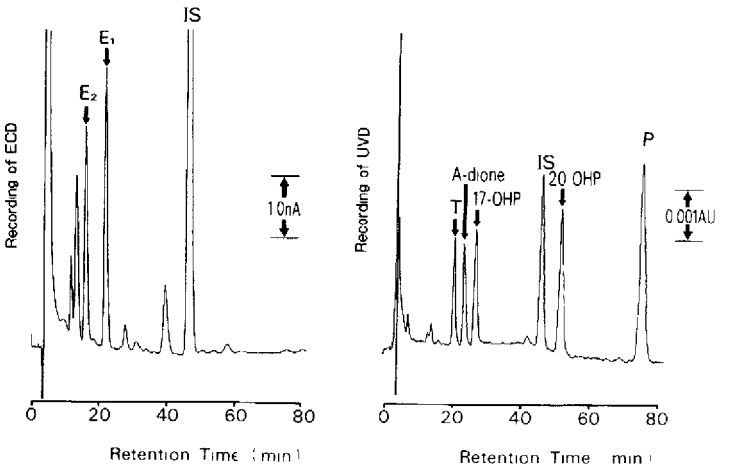

Fig. 2. Chromatograms of the authentic steroid hormones and the internal standard in 0.2 ml of serum (at the time of menstruation). E_2 = estradiol, 9.6 ng; E_1 = estrone, 13.4 ng; IS = 4′-methoxyoctananilide, 100.0 ng; T = testosterone, 25.6 ng; A-dione = androstenedione, 23.5 ng; 17-OHP = 17α-hydroxyprogesterone, 29.8 ng; 20-OHP = 20α-hydroxyprogesterone, 52.0 ng; P = progesterone, 98.7 ng; ECD = electrochemical detector; UVD = ultraviolet spectrophotometric detector.

hormones thus eluted were introduced into the analytical column and separated with the same eluent. E_2 and E_1 were detected electrochemically and T, A-dione, 17-OHP, 20-OHP and P were detected by UV absorption.

The pretreatment column was regenerated by washing with water and methanol for 10 min. The next sample could then be applied to the column while the previous sample was on the analytical column.

RESULTS

Basic study

The chromatogram of a mixture of steroid hormone standards and I.S. detected electrochemically are shown in Fig. 2. E_2, E_1 and I.S. were well separated and their retention times were 15.8, 21.6 and 46.0 min, respectively. T, A-dione, 17-OHP, I.S. 20-OHP and P were also well separated and detected by UV absorption: their retention times were 20.5, 23.2, 26.7, 45.9, 51.6 and 75.5 min, respectively. The detection limits of authentic samples with this apparatus, at a signal-to-noise ratio of 3, were 50 pg for E_2 and E_1, 0.5 ng for T, A-dione and 17-OHP, 1.0 ng for 20-OHP and 1.5 ng for P. However, in measurements of serum samples, small unknown peaks were detected by UV absorption, especially near the peaks of T, A-dione and 17-OHP. Therefore, in routine determination the measurable limits of E_2 and E_1 were ca. 250 pg/ml, those of T, A-dione and 17-OHP were ca. 5.0 ng/ml and those of 20-OHP and P were ca. 5.0 and 7.5 ng/ml, when 0.2 ml of serum was applied.

The relationship between the dose of each sex steroid hormone and the peak height showed good linearities in the ranges from 0.1 to 20 ng for E_2 and E_1 and from 2 to 80 ng for T, A-dione, 17-OHP, 20-OHP and P.

From study of the chromatogram of standards and that of standards in serum (at the time of menstruation) obtained by our method, the recovery rates of E_2, E_1, T, A-dione, 17-OHP, 20-OHP and P were 84.0 ± 1.8, 83.7 ± 2.3, 81.1 ± 1.6, 77.0 ± 2.4, 77.5 ± 1.3, 79.7 ± 2.0 and $81.6 \pm 1.9\%$, respectively. The intra-assay coefficients of variation (CV) of the peak height of E_2, E_1, T, A-dione, 17-OHP, 20-OHP and P were 2.1, 2.8, 2.0, 3.1, 1.7, 2.4 and 2.1%, respectively ($n=10$).

Clinical application

Fig. 3A shows a chromatogram obtained from a maternal serum at the 38th week of pregnancy before loading DHAS. The levels of E_2, E_1, 20-OHP and P were 25.5, 14.7, 24.2 and 74.2 ng/ml, respectively. Fig. 3B shows a chromatogram obtained from the mother's serum 30 min after intravenous infusion of 50 mg of DHAS. E_2 was 280%, E_1 was 137%, 20-OHP was 101% and P was 95% of preinfusion levels.

In pregnant women, the correlation between E_2 values obtained by the present HPLC method and by RIA was significant ($r=0.938, p<0.001$), as shown in Fig. 4A. The correlation between P values obtained by our method and by RIA was good ($r=0.903, p<0.001$), as shown Fig. 4B.

Fig. 5 shows a chromatogram obtained from 25 μl of ovarian follicular fluid of the preovulatory follicle after ovarian stimulation, when the follicle size checked by an ultrasonograph (Type SSD 280, Aloca, Tokyo, Japan) was $20 \times 20 \times 15$ mm. E_2, E_1, T, A-dione, 17-OHP, 20-OHP and P were 321, 11.2, 225, 59.2, 561, 296 and 6030 ng/ml, respectively.

DISCUSSION

E_2, E_1, T and A-dione in the serum bind mainly albumin and sex hormone binding globulin (SBG), and P, 17-OHP and 20-OHP in serum bind mainly albumin and corticosteroid binding globulin (CBG) [4]. To measure unconjugated sex steroid hormones, including the bound forms, we applied competitive binding between these steroids and DHT and F, since DHT has the greatest binding capacity to SBG and F has the greatest binding capacity to CBG. We assumed that 5 μg each of DHT and F were sufficient to liberate bound steroids from respective binding proteins when 0.2 ml of the serum was used, because the peaks of steroids in any pregnant woman's serum were not elevated even if the amounts of DHT and F increased above this level. Binding protein levels in serum are lower in non-pregnant than in pregnant women [5], and those in follicular fluid are either similar to or lower than those in serum [6]. Thus, these amounts of DHT and F seem to be sufficient in almost all samples. DHT was detected only by UV absorption between the peak of 17-OHP and that of I.S., but its peak was hardly detectable at a wavelength of 245 nm because the maximum absorption wavelength of DHT is 209 nm. F was also detected only by UV absorption, and the peak of F was hidden in the front unknown peak. Thus DHT and F do not interfere with the present method.

In the present study, peaks of 20-OHP and A-dione were identified. It is difficult to separate E_2 chromatographically from T and E_1 from A-dione and 17-OHP

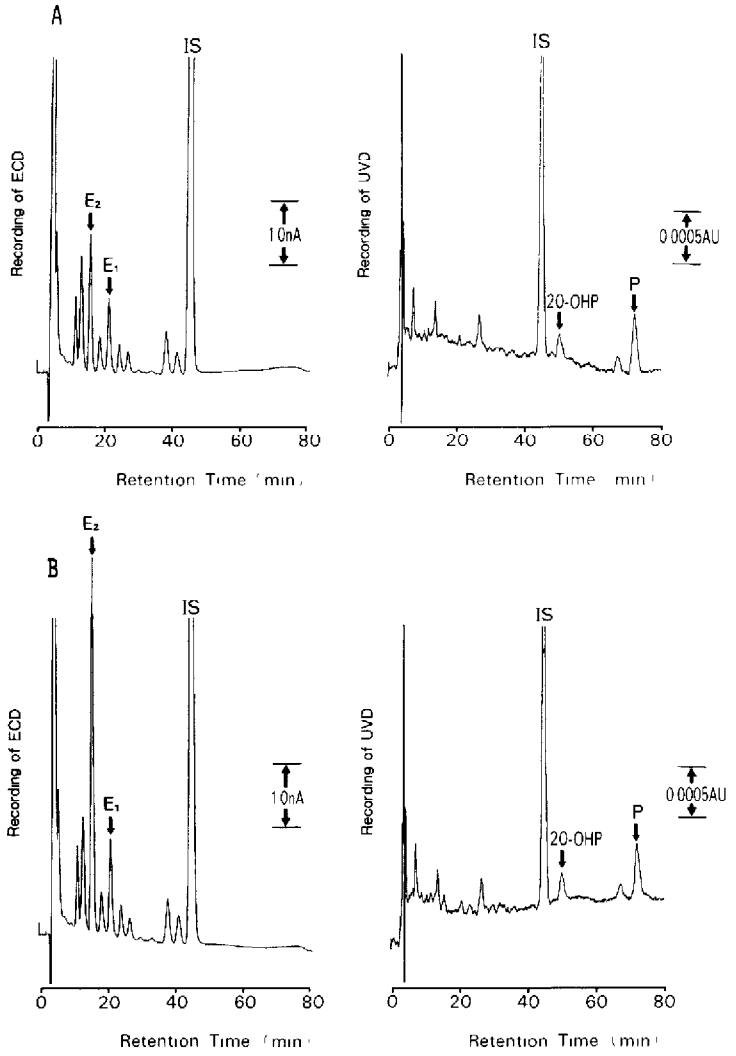

Fig. 3. (A) Chromatogram obtained from 0.2 ml of a maternal serum at the 38th week of pregnancy before loading DHAS. Concentrations: $E_2 = 25.5$ ng/ml; $E_1 = 14.7$ ng/ml; 20-OHP = 24.2 ng/ml; P = 74.2 ng/ml. (B) Chromatogram obtained from 0.2 ml of the mother's serum 30 min after infusion of 50 mg of DHAS. Concentrations: $E_2 = 71.5$ ng/ml; $E_1 = 20.1$ ng/ml; 20-OHP = 24.5 ng/ml; P = 70.7 ng/ml.

[7]. However, the complete separations of these hormones were obtained with the use of two specific detectors in our chromatograms, as shown in Fig. 2 and Fig. 5.

The re-usability of the pretreatment column was studied by repeated measurements on the same column. We used a Separapid tube to remove fibrin completely from a sample, and used a filter in front of the pretreatment column. The flow direction of the column was reversed during washing with methanol and water; the pressure of the column did not increase and the column could be used for the pretreatment at least 100 times.

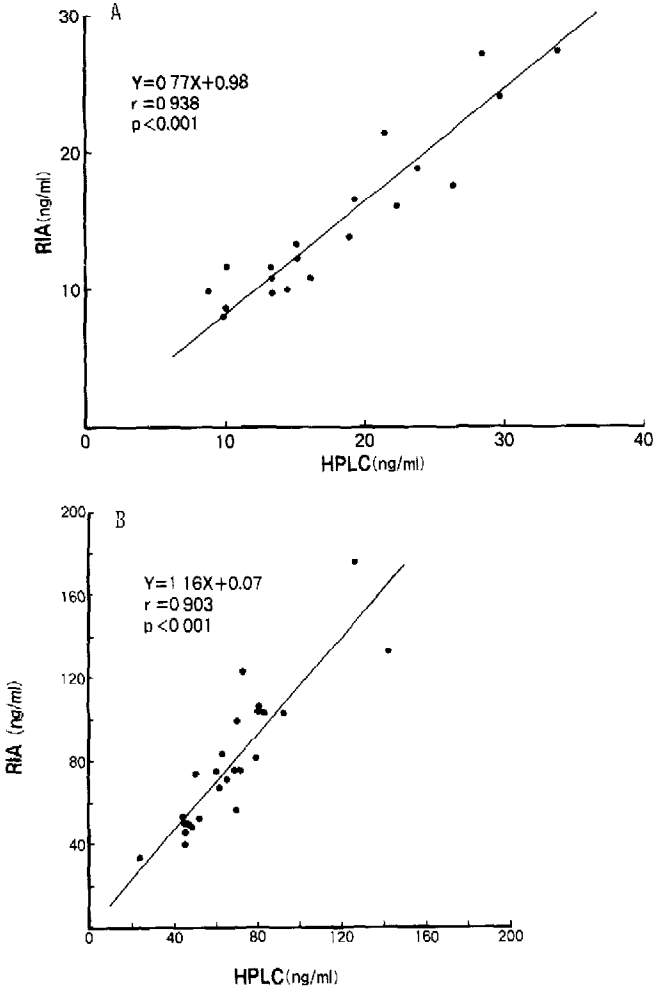

Fig. 4. (A) Correlation between E_2 concentrations obtained by the present HPLC method and those obtained by RIA. (B) Correlation between P concentrations obtained by our method and those obtained by RIA. Samples of maternal sera from subjects in the 18th to 39th week of pregnancy.

E_2, E_1 and P are useful as the indices of placental function, and 20-OHP is a metabolite of P. The maternal serum estrogen response to injected DHAS reflects placental function. Tulchinsky et al [8] reported a rise (ca. 280–370%) in the level of E_2 following the infusion of DHAS in normal pregnant women at 35–40 weeks of gestation. In the case shown in Fig. 3, the increase was 280%. However, the monitoring of these hormone levels has not been generally used in clinical practice, since the results could not be obtained rapidly. The present method does give rapid results. Moreover, the profiles of these hormones may be useful for the assessment of clinical status.

Ovarian follicular sex steroid concentrations (E_2, P and A-dione) have been reported as indicators of follicular maturation, since a greater concentration of

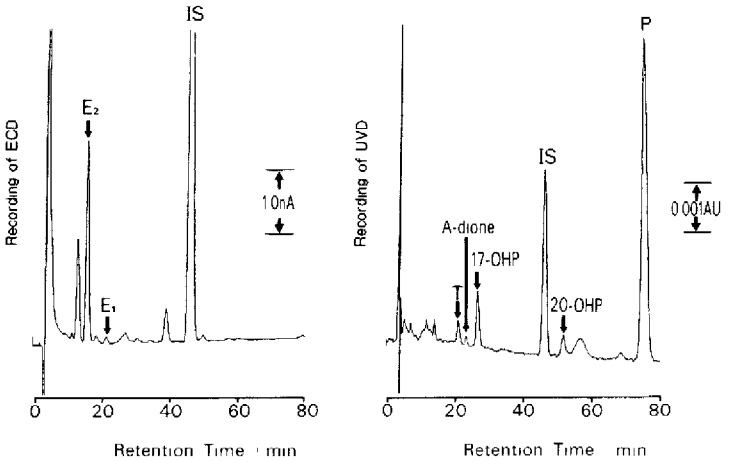

Fig. 5. Chromatogram obtained from 25 μl of ovarian follicular fluid at the preovulatory period in ovulation induction therapy, when the follicle size checked by ultrasonograph was 20×20×15 mm. Concentrations: E_2 = 321 ng/ml; E_1 = 11.2 ng/ml; T = 225 ng/ml; A-dione = 59.2 ng/ml; 17-OHP = 561 ng/ml; 20-OHP: 296 ng/ml; P = 6030 ng/ml.

E_2 and a higher ratio of E_2 to P are associated with successful fertilization and pregnancy [9]. As shown in Fig. 5, the peaks of the sex steroids in follicular fluid are very high. Therefore, the present method might also be useful in the practice of in vitro fertilization and embryo transfer.

Employing this method, we could also monitor the follicular maturation and luteal function as reported previously [1] and analyse steroid hormones in other biological fluids than serum, such as amniotic and ascites fluids. Thus the present method has wide applicability in clinical practice, since results can be obtained rapidly and simultaneously.

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