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Rapid and simultaneous measurement of estrone, estradiol, estriol and estetrol in serum by high performance liquid chromatography with electrochemical detection.

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Abstract

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RAPID AND SIMULTANEOUS MEASUREMENT OF ESTRONE, ESTRADIOL, ESTRIOL AND ESTETROL IN SERUM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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Abstract. A high performance liquid chromatographic (HPLC) method with electrochemical detection (ECD) was developed for the simultaneous measurement of estrone, estradiol, estriol and estetrol in serum. These hormones were extracted with diethylether, chromatographed on an silica-octadecyl silane (ODS) column with an eluent of phosphate buffer solution-acetonitrile-methanol (volume ratio 152 : 85 : 40), and detected by ECD at + 1.0 V vs. Ag/AgCl. In comparisons between the values measured by this method and radioimmunoassay, significant correlations were noted for estrone ($r = 0.759$, $p < 0.01$), estradiol ($r = 0.816$, $p < 0.001$) and estriol ($r = 0.830$, $p < 0.001$). In clinical applications of this method, differences between cases of the normal and the anencephalic pregnancy in the thirty-eighth week of gestation were distinct not only in the individual estrogen, but also in the profile analysis of estrogens. With this method, all 4 serum estrogens above approximately 500 pg/ml could be measured within 2 h, and the method seemed to be clinically applicable.

Key words : high performance liquid chromatography, electrochemical detection, serum estrogens.

It has become clear that unconjugated estrogens (estrone, estradiol, estriol and estetrol) in maternal serum are useful as indices of fetal and placental function. Estriol is especially useful since its biosynthesis mainly participates in fetal and placental functions. The biosyntheses of estrone and estradiol mainly participate in placental function, and that of estetrol in fetal function.

As it is very difficult to obtain results rapidly with radioimmunoassay, the development of a new method of rapid determination has become necessary. For this purpose, a method which combines single-elution HPLC with ECD has been developed to measure unconjugated estrogens in maternal serum.

MATERIALS AND METHODS

The standard estrogens were the products of Sigma (Missouri). The internal standards, ρ-methoxy-n-valeric anilide (MVA) and ρ-methoxy-n-caproic anilide (MCA), were obtained from Eisai (Tokyo). Primary potassium hydrogen phosphate and all of the organic solvents

were the products of Katayama Chemical Ind. Co., Ltd. (Osaka). Acetonitrile, methanol and n-hexane were HPLC grade, and diethylether was first grade.

A shaker (Yayoi YFS-D) and a centrifugal-evaporator (Yamato RD-21) were used for preparation of samples. A chromatograph (Yanaco L-2000), an electrochemical detector (Yanaco VMD-101) and an oven for the electrochemical detector (Yanaco LA-100) were used for analysis. The column used for separation was an ODS column (Yanapak ODS-T, 4 mm in inner diameter by 250 mm in length).

A known amount of MVA and MCA were added as internal standards to 1 ml of serum. Extraction 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℃ 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 μl of methanol and 20 μl of the solution was used for the analysis. The time required for this preparation was about 50 min for one sample, and about 2 h for simultaneous treatment of 10 samples.

The eluent of HPLC was studied, keeping the flow rate constant at 1.0 ml/min and the temperature of the oven at 25.0 ± 0.1℃. First, the following 3 eluent systems were studied: the phosphate buffer solution-acetonitrile system, phosphate buffer-methanol system and phosphate buffer solution-acetonitrile-methanol system (Fig. 1).

The order of elution for estrogens using the phosphate buffer solution-acetonitrile system, was estetrol, estriol, estradiol and estrone. With male serum, admixtures tended to gather

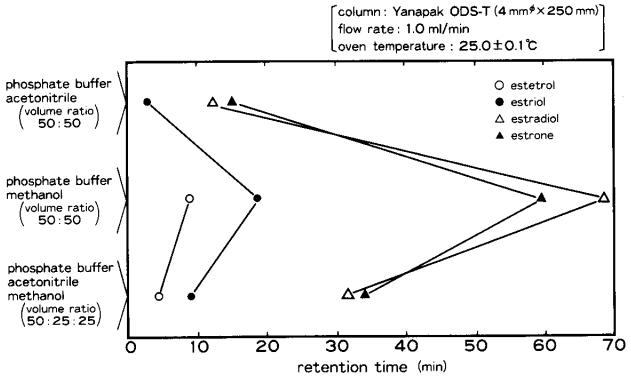

Fig. 1. The effect of eluent on retention time of setrogens.

around estetrol and estriol. When the proportion of acetonitrile was reduced in order to separate estetrol and estriol from the admixtures, the retention times of estradiol and estrone were prolonged. This system did not seem advantageous for the simultaneous analysis of all 4 estrogens, but it was useful for the analysis of estradiol and estrone.

The order of elution using the phosphate buffer solution-methanol system, was estetrol, estriol, estrone and estradiol. Compared with the phosphate buffer solution-acetonitrile system, the order of elution was reversed for estradiol and estrone, and this system showed a longer retention time for each estrogen. With male serum, admixtures tended to gather around estrone and estradiol. This system seemed advantageous for the analysis of estetrol

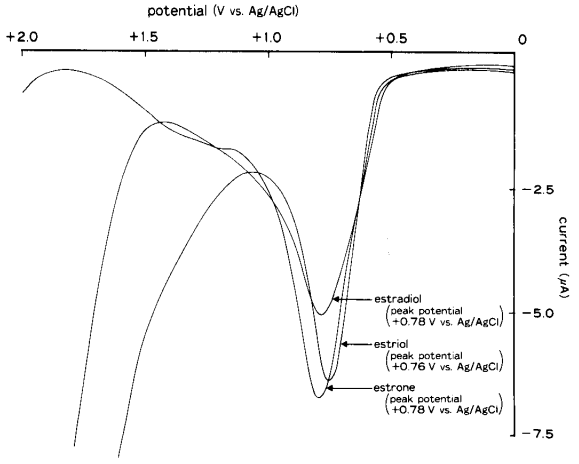

Fig. 2. Electrolytic reaction of estrogens in the phosphate buffer - acetonitrile - methanol mixture versus the Ag/AgCl electrode with the differential pulse polarograph.

and estriol.

When acetonitrile and methanol were mixed with a phosphate buffer solution at the ratio of acetonitrile/methanol $\geqq$ 1.0, the order of elution was estetrol, estriol, estradiol and estrone. It was possible to obtain good separation even with shortening the total elution time, reducing the interference of admixtures of male serum in the analysis of estrogen by adjusting the composition ratio. This system was advantageous for the simultaneous analysis of the 4 estrogens.

From these studies, the optimum composition of the eluent was determined to be phosphate buffer-acetonitrile-methanol at a volume ratio of 152 : 85 : 40.

Next, the influence of changing the concentration of phosphate buffer solution on the analysis of estrogens was studied with 0.1, 0.05 and 0.025 M primary potassium phosphate solutions. The retention time of each estrogen did not change with a change in concentration. As a slight decrease in the height of each estrogen peak was noted in the case of 0.025 M, the 0.05 M primary potassium hydrogen phosphate solution, which gave no trouble in either the analysis or the flow passage, was used.

Phosphoric acid was added dropwise to the 0.05 M primary potassium phosphate solution to prepare phosphate buffer solutions at pH 2.6, 3.1, 3.6, 4.1 and 4.6. The change in pH produced practically no change in the retention times of estetrol and estriol, while those of estradiol and estrone were changed. In the case of pH 3.1, the retention time was shortest, which was advantageous for the analysis.

In order to determine the optimum potential applied to the ECD electrolytic system, the electrolytic reactions of estrone, estradiol and estriol versus the Ag/AgCl electrode in the solution chosen as the eluent, were studied with a differential pulse polarograph (Yanaco P-1000), as shown in Fig. 2. The peak potentials of each estrogen was +0.76 to +0.78 V vs. Ag/AgCl, and the current limits were reached at +1.0 V vs. Ag/AgCl, so the potential was set at +1.0 V vs. Ag/AgCl. The amplified change of current was recorded at the sensitivity of 1 nA to 32 nA full scale and the chart speed of 0.5 cm/min.

A methanol solution of the mixture of standard estrogens was prepared in known quantities and applied directly to HPLC-ECD to measure the retention times, the sensitivities of de-

tection and the calibration lines. Additionally, estrogen standards (estrone: 10.88 ng, estradiol: 8.03 ng, estriol: 6.00 ng, estetrol: 5.25 ng), and MVA (7.91 ng) and MCA (10.44 ng), were added to male serum (n = 5). These were applied to HPLC-ECD after preparation to examine the recovery and reproducibility of this method, and the applicability of MVA and MCA as internal standards.

Maternal serum, obtained from each one case of normal and anencephalic pregnancy in 38th week of gestation, were analyzed by this method. Moreover, 14 samples of maternal serum from subjects in the 24th to 40th weeks of gestation were analyzed by both this method and radioimmunoassay (entrusted to Teikoku Zouki Pharm. Co., Ltd.), and the measured values were compared.

RESULTS

Preliminary study. Each estrogen was separated and detected satisfactorily by HPLC-ECD, as shown in Fig. 3. The retention times of estetrol, estriol, estradiol

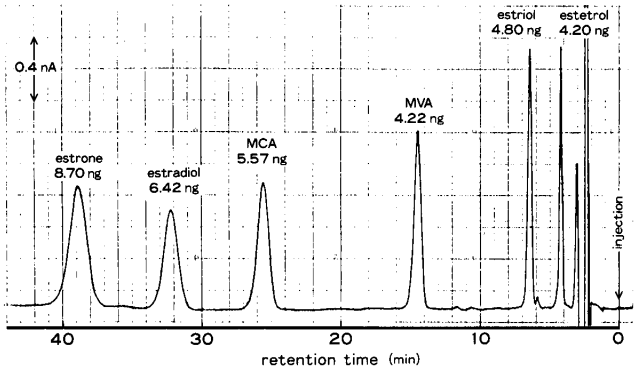

Fig. 3. Chromatogram of standard estrogens and internal standards

and estrone were 4.2, 6.6, 32.2 and 38.9 min, respectively. These retention times were constant, and the total time required for the analysis was 42 min.

A stable baseline on the chromatogram was obtained with sensitivities of up to 1 nA full scale, and the detection limits with this apparatus (signal/noise ratio = 5) for estetrol, estriol, estradiol and estrone were 46, 49, 133 and 137 pg, respectively.

The relationship between the dose of each estrogen and the peak height detected on the chromatogram was linear in the range from 100 ng to about 150 pg, as shown in Fig. 4.

From the study of male serum to which standard estrogens were added, the average recoveries of estetrol, estriol, estradiol and estrone were 65, 87, 85 and 79 %, respectively. The C.V. values of the peak height of estetrol, estriol, estradiol and estrone were 4.9, 2.1, 2.4 and 2.7 %, respectively. These results showed that the recovery and the reproducibility of this method were excellent.

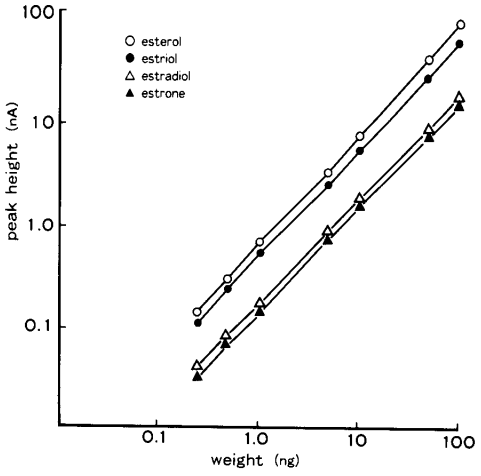

Fig. 4. Calibration line

MVA and MCA showed good separation from each estrogen, as shown in Fig. 3. The retention times of MVA and MCA were 14.5 and 25.6 min, respectively. The detection limits with this apparatus of MVA and MCA were 65 and 121 pg, respectively. From the study of male serum, the average recoveries of MVA and MCA were 64 and 80 %, respectively, and the C.V. values of MVA and MCA were 3.0 and 2.6 %, respectively. In this way, MVA and MCA showed sensitivities nearly equal to those of estrogens, with good recovery and reproducibility. They did not prolong the total time required for the analysis, and made determination possible with high precision. Compared to MVA, MCA is superior in its recovery and reproducibility, so it was used primary.

Clinical application and comparison with radioimmunoassay. Fig. 5 shows chromatograms obtained from 1 ml of maternal serum from each one case of normal and anencephalic pregnancy in the 38th week of gestation with standard estrogens in known quantities added to 1 ml of water. Corresponding to each standard estrogen, a peak was formed at the expected retention time with no distortion. It was clearly shown that the estriol peak was much lower in the anencephalic case than in the normal case. Furthermore, not only in the peaks of individual estrogen, but also in the profile of the 4 estrogen peaks, there were distinct differences between normal and anencephalic cases. The measured values for each estrogen (quantified with the internal standard, measuring the peak height) were estetrol: 0.50 ng/ml, estriol: 18.21 ng/ml, estradiol: 29.75 ng/ml, and estrone: 8.68 ng/ml in the normal case, and estetrol: not detectable, estriol: 1.01 ng/ml, estradiol: 9.26 ng/ml and estrone: 3.64 ng/ml in the anencephalic case.

Table 1 shows a comparison between the values measured by this method and those measured by radioimmunoassay of 14 samples of serum from subjects in the 24th to 40th weeks of gestation. These were significant correlations in estrone (r

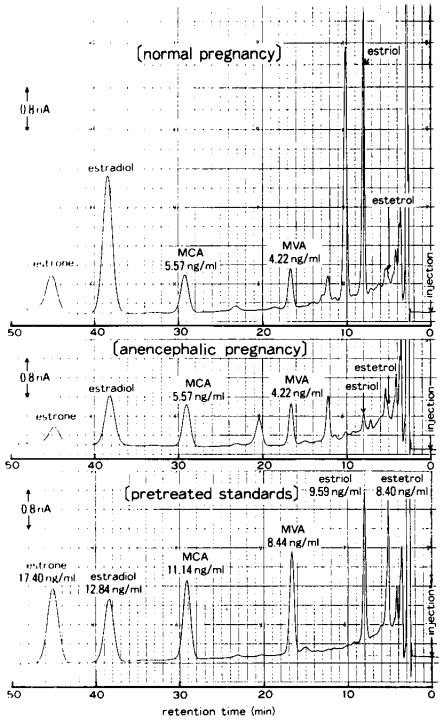

Fig. 5. Chromatograms obtained from 1 ml of maternal serum from normal and anencephalic pregnancies in the 38th week of gestation with standard estrogens in known quantities added to 1 ml of water.

TABLE 1. ESTROGEN VALUES OF MATERNAL SERUM BY HPLC AND RADIOIMMUNOASSAY

Gestation (week)	Estrone (ng/ml)		Estradiol (ng/ml)		Estriol (ng/ml)		Estetrol (ng/ml)	
	HPLC[a]	RIA[b]	HPLC	RIA	HPLC	RIA	HPLC	RIA
24	9.38	7.71	15.59	8.55	1.68	2.67	n.d.	0.07
25	5.92	6.59	8.25	5.52	3.45	3.93	n.d.	0.18
26	10.36	6.79	8.25	7.82	3.26	3.18	n.d.	0.11
27	6.91	4.43	10.09	6.79	3.17	3.29	n.d.	0.10
28	5.43	4.56	7.34	6.16	4.10	3.55	n.d.	0.11
29	15.80	15.40	10.55	10.60	4.10	5.54	n.d.	0.19
30	7.90	5.28	6.88	7.80	4.19	5.03	n.d.	0.19
31	6.41	5.09	10.55	8.51	4.94	4.75	n.d.	0.23
32	15.30	5.34	16.05	15.90	4.75	4.87	n.d.	0.36
34	13.82	7.16	16.51	18.80	11.74	7.90	n.d.	0.33
35	15.80	12.80	16.51	19.00	13.79	4.53	0.83	0.31
37	5.92	3.25	14.68	14.10	9.32	8.61	1.67	0.49
39	4.44	3.72	8.71	11.50	7.46	6.26	0.42	0.55
40	11.85	8.97	20.18	16.10	17.90	14.10	1.25	0.56

[a]: High performance liquid chromatography

[b]: Radioimmunoassay

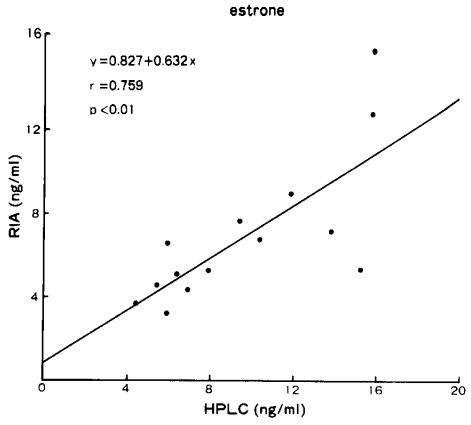

Fig. 6. Correlation of estrone values between HPLC and radioimmunoassay.

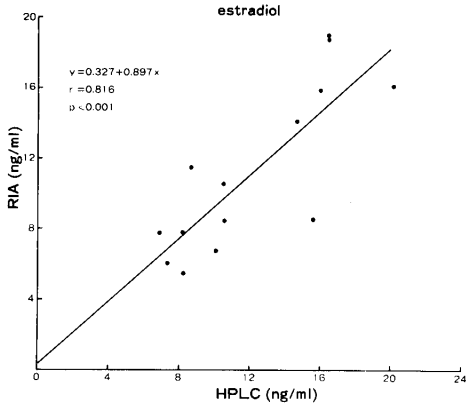

Fig. 7. Correlation of estradiol values between HPLC and radioimmunoassay.

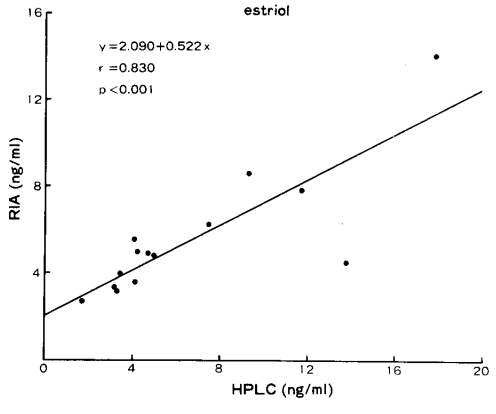

Fig. 8. Correlation of estriol values between HPLC and radioimmunoassay.

$= 0.759$, $p < 0.01$), estradiol ($r = 0.816$, $p < 0.001$) and estriol ($r = 0.830$, $p < 0.001$), as shown in Figs. 6, 7 and 8. Concerning estetrol, it was possible to measure it in cases after the 35th week of gestation by this method, but no significant correlation was noted. Compared with radioimmunoassay, the values measured by this method tended to be slightly higher.

In clinical practice, the detection limits of estrogens in serum were approximately 500 pg/ml.

DISCUSSION

Estrogens in maternal urine are used clinically as an index of feto-placental function, various simplified methods having been developed (1-3). However, rapid clinical evaluation is difficult when urine is examined, since it must be accumulated for 24 h in principle. Additionally, estrogens in the urine are final metabolic products. On the other hand, estrogens in maternal serum reflect fetal status more closely. Estriol, estradiol and estrone in maternal serum are biosynthesized mainly in the placenta (4). Concerning estrone and estradiol, the degree of dependence on precursors is considered to be about 40 % on the maternal side, and about 60 % on the fetal side (5. 6), while concerning estriol, the degree of dependence on precursors is considered to be about 10 % on the maternal side and 90 % on the fetal side (7, 8). Furthermore, estetrol is considered to be biosynthesized in the fetal liver from estradiol in fetal serum (9, 10). Thus, the biosyntheses of estrone, estradiol, estriol and estetrol are all related specifically to fetal and placental functions. In addition, estrogens in serum are rapidly cleared; the half-life of estriol is about 20 min (11). Moreover, they have smaller daily variations compared to estrogens in urine collected during 24 h. Twenty-four hour urinary estriol has a coefficient of variation of 21 %, whereas the day to day variation of plasma estriol is 13 % (12). Thus, the measurements of these estrogens are extremely important in the evaluation of fetal and placental functions, which are changing every second.

At present, radioimmunoassay is used generally for the measurements of estrogens in serum because of its sensitivity, but the results of measurement cannot be obtain rapidly, and special facilities and equipment are needed to handle radioactive substances. Consequently, it is not always a suitable method for clinical use.

Recently, a method has been developed to measure $\triangle$4-3 ketosteroid hormones in serum using HPLC, which can be used clinically to measure several steroids simultaneously and rapidly (13, 14). Ultraviolet detection used generally in combination with HPLC is not sufficiently sensitive for estrogens. Since Kissinger *et al.* (15) applied ECD to the measurement of catecholamine in combination with HPLC in 1973, HPLC-ECD has been applied to the measurement of substances with electrochemical characteristics. In its application to the analysis of estrogens, Shimada *et al.* (16) reported the method of measuring catechol estrogen in urine, Hiroshima *et al.* (17) the method of measuring estriol in urine, and Sagara *et al.* (18)

the method of measuring estrone, estradiol, estriol and estetrol in serum. Sagara *et al.* determined estrogens, but they separated estrone and estradiol from estriol and estetrol by column chromatography, before the determination by HPLC-ECD (18). In our system, estrone, estradiol, estriol and estetrol are separated by a single elution method.

Compared with radioimmunoassay, the time required for the measurement of one sample is shorter. However, a long time is required to measure a number of samples and sufficient concern for the analytical conditions is necessary. For example, a certain quantity of solution must always be mixed in a certain container, and the time for the removal of air should be constant. Furthermore, changes in temperature influence the analysis greatly. The elevation of the oven temperature shortened the retention time and increased the peak height. These changes were the most marked with estrone, which showed the longest retention time. When the temperature of the oven was changed from 25.0℃ to 27.5℃, the retention time of estrone changed about 3 min and the height of peak about 10 %. For this reason, it was indispensable to set the temperature constant.

However, even if the conditions for the analysis are maintained strictly in this way, deterioration of the column and the detector cell are inevitable, and it is a problem that the determination is performed only by means of a calibration line. Additionally, in clinical application it is necessary to improve the reliability by correcting the recovery for each sample. For this purpose, we studied the application of the fatty acid derivative of *ρ*-methoxyaniline, which was used by Hiroshima *et al.* as an internal standard to measure estriol in urine (17). We demonstrated that determination with high precision was possible without prolonging the total time required for the analysis if MCA or MVA were used.

The estrone, estradiol and estriol values measured by this method and radioimmunoassay showed significant correlations, as shown in Figs. 6, 7 and 8. Thus, it was demonstrated that qualitative and quantitative measurements by this method are highly reliable. The content of estetrol in the serum is relatively small, and the estetrol peak was close to the front peak, causing some problems in its measurement. By this method, measurement was possible with a sample after the 35th week of gestation, but the number of such cases was few, and no correlation was noted with the values measured by radioimmunoassay. The measurement of estetrol must be studied further.

In this study, a woman with anencephalic fetus had a lower estriol value than a woman with normal pregnancy, reflecting the pericular endocrinopathic state of fetal adrenal atrophy. This corresponds to the report of Nakayama *et al.* (20), using radioimmunoassay. Thus, the measurement of estriol with this method was considered useful for the screening of anencephalic pregnancy.

However, clinical states are extremely complex and varied, and estrogen levels are influenced by many factors other than fetal and placental functions. These

are fetal weight, maternal intestinal flora, maternal renal function and maternal liver function (11, 12, 19). Therefore, the evaluation of fetal and placental functions and the understanding of pathological conditions might be incomplete with a single estrogen measurement as an index. In order to evaluate and understand more precisely, it seems important to perform profile analysis of estrone, estradiol, estriol and estetrol. In the comparison between cases of the normal and the anencephalic pregnancy in this study, the differences were distinct not only in the individual estrogen, but also in the pattern of the four estrogens, as shown in Fig. 5. Moreover, the profile analysis is considered important to prevent potentially grievous clinical situations, such as a wrong clinical judgment based on incorrect measurements. This method is excellent for profile analysis, as the separation and detection are performed consistently.

The simultaneous measurements of estrone, estradiol, estriol and estetrol are possible within about 2 h, which was suitable for clinical use.

The main points of this article were presented at the 33rd Annual Meeting of the Japanese Society of Obstetrics and Gynecology (Niigata, May 1981) and the 54th Annual Meeting of the Japan Endocrine Society (Okayama, June 1981).

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