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Automated direct high-performance liquid chromatographic assay for estetrol, estriol, cortisone and cortisol in serum and amniotic fluid[a]

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ABSTRACT

An automated direct assay for the simultaneous determination of unconjugated estetrol, estriol, cortisone and cortisol in serum and amniotic fluid, using high-performance liquid chromatography with electrochemical detection and ultraviolet detection, has been developed. The analysis time is *ca.* 1 h. This system offers good reproducibility with low coefficients of variation (estetrol, 2.3%; estriol, 2.3%; cortisone, 2.6%; cortisol, 1.9%). Detection limits are low enough for routine determinations (estetrol and estriol, 150 pg; cortisone and cortisol, 5 ng). Comparison of the values measured by the present method and by radioimmunoassay revealed significant correlations for estetrol ($r=0.787$, $p<0.01$), estriol ($r=0.957$, $p<0.01$), cortisone ($r=0.956$, $p<0.01$) and cortisol ($r=0.865$, $p<0.01$). This system proved to be valuable in monitoring feto-placental function.

INTRODUCTION

Urinary estriol (E_3) levels have been used for evaluating feto-placental function during the third trimester of gestation. Thereafter, unconjugated estetrol (E_4) and E_3 in serum are coming to be recognized as more sensitive indicators of the feto-placental function, on the basis of their biosynthetic pathway [1,2]. Glucocorticoids were reported to be related to estrogen levels in maternal serum [3], fetal lung maturation [4] and initiation of labour [5].

Assays of unconjugated estrogens and glucocorticoids in biological fluids have been usually carried out by radioimmunoassay (RIA), which requires complex procedures [6,7]. Recently, Suzuki *et al.* [8] devised an automated direct assay for measuring estradiol (E_2), estrone (E_1), progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, testosterone and androstenedione using high-performance liquid chromatography (HPLC). This paper describes an automated

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direct assay for measuring unconjugated E_4, E_3, cortisone (E) and cortisol (F) in biological fluid.

EXPERIMENTAL

Samples

All samples of serum and amniotic fluid obtained were kept in Separapid tubes (Sekisui Chemical Industries, Osaka, Japan) for *ca.* 10 min to remove the fibrin. The tubes were centrifuged at 1000 *g* for 10 min, and the supernatants were placed in glass tubes and stored at −20°C until analysis.

Reagents

The standard estrogens, glucocorticoids, the internal standards {1,3,5[10]-estratriene-3,16α-diol-17-one (16-E_1) and 1,4-pregnadiene-17α,21-diol-3,11,20-trione (Pd)} were purchased from Sigma (St. Louis, MO, USA). Potassium dihydrogenphosphate and the organic solvents were from Katayama Chemical Industries (Osaka, Japan). Acetonitrile and methanol were HPLC grade. Phosphate buffer was prepared by dissolving 6.8 g of potassium dihydrogenphosphate in 1 l of distilled water, and the pH adjusted to 3.1 with concentrated phosphoric acid.

Apparatus

The present system is a modification of that of Suzuki *et al.* [8]. The pretreatment unit was equipped with a chromatographic pump (MS-610, Sekisui, Osaka, Japan), a pretreatment column (Serumout-25, Sekisui) and an autosampler (ASU-420, Sekisui). The analytical unit was equipped with a chromatographic pump (LCP-320, Sekisui), an analytical column (Medipola-ODS, C_{18}, 5 μm, 260 mm × 4.6 mm I.D., Sekisui), an electrochemical detector (ECD-120, Sekisui), a UV detector (UVD-121, Sekisui), a degasser (ERC-3310, ERMA, Tokyo, Japan) and a data-processor (Type 7000B, System Instruments, Tokyo, Japan). A column oven (COU-820, Sekisui) was used to keep the two columns at 40.0°C.

Chromatographic procedure

An aliquot (0.5–1.0 ml) of each biological fluid was diluted with 500 μl of 5% methanol solution containing 16-E_1 (50.5 ng) and Pd (75 ng), and 750 μl of this solution were applied to the system.

E_4, E_3, E, and F in a sample were adsorbed on the pretreatment column, which was washed for 5 min with distilled water at a flow-rate of 0.8 ml/min. Then the pretreatment column was incorporated into the analytical unit for 5 min, by switching the six-way electromagnetic valve. These hormones were eluted from the pretreatment column and then separated on the analytical column with a mobile phase of phosphate buffer–acetonitrile–methanol (20:2:7, v/v) at a flow-rate of 1.0 ml/min. E_4 and E_3 were detected electrochemically at +1.0 V *vs.* Ag/AgCl. E and F were detected at a UV wavelength of 245 nm.

RESULTS

Pure standards of estrogens, glucocorticoids, internal standards, several possible interfering substances and serum samples were applied to the system. The retention times of E_4, E_3, 16-E_1, Pd, E and F were 15.6, 31.6, 52.4, 50.8, 56.8 and 66.0 min, respectively. Pure standards of 16α-epiestriol (16-E_3), E_2 and E_1 detected electrochemically had retention times more than 52.4 min. Pure standards of corticosterone, testosterone, 17α-hydroxyprogesterone, androstenedione and progesterone detected spectrophotometrically had retention times of more than 66 min. The conjugated compounds of those steroids on the pretreatment column were eluted by distilled water. They did not interfere with this assay. As shown in Fig. 1, E_4, E_3, E, F and the internal standards were separated and detected satisfactorily by the present system. Minimum detectable amounts (signal-to-noise ratio 3) for E_4, E_3, E and F were 82 pg, 187 pg, 5.8 ng and 7.8 ng, respectively. The relationship between the amount of each steroid and the peak area on the chromatogram was linear in the following ranges: E_4, 0.082–21.00 ng; E_3, 0.19–47.90 ng; E, 5.8–188.4 ng; F, 7.8–250.0 ng. The average recoveries (n = 6) of E_4, E_3, E and F were 104.8, 99.4, 99.5 and 91.2%, respectively (Table I). The within-run coefficients of variation (C.V.) for E_4, E_3, E and F were 1.9, 2.9, 0.7

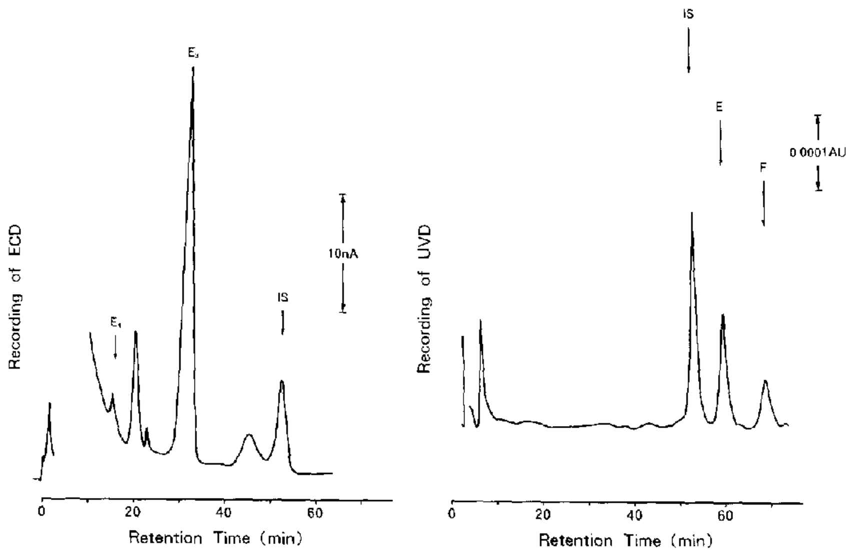

Fig. 1. Chromatograms obtained from the umbilical vein fluid of a woman at 39 weeks gestation. The first 2–10 min of the chromatograms correspond to the front peak. The front peaks in this system are wider than those from a single injection, for the volume of pretreatment column. They originate from mixing of the carrier and distilled water from the pretreatment column; thus, wide front peaks are also observed for pure samples. IS = 1,3,15[10]-estratriene-3,6α-diol-17-one and 1,4-pregnadiene-17α,1-diol-3,11,20-trione. Concentrations: E_4 (estetrol), 2.07 ng/ml; E_3 (estriol), 102.83 ng/ml; E (cortisone), 169.87 ng/ml; F (cortisol), 63.29 ng/ml.

TABLE I

RECOVERIES OF E_4, E_3, E AND F

a = Expected value (ng); b = measured value (ng).

Sample No.	E_4		E_3		E		F	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
1	7.9	7.8	50.1	49.8	100.6	94.3	95.2	84.5
2	4.1	4.1	41.1	37.5	67.0	64.1	50.5	45.3
3	2.3	2.2	36.9	33.4	50.0	51.3	28.2	26.9
4	8.1	8.9	49.5	53.1	97.5	94.2	95.2	86.4
5	4.3	4.9	40.0	44.1	63.8	63.7	50.6	45.7
6	2.4	2.6	36.6	36.7	47.0	51.2	28.2	26.1
Total	104.8%		99.4%		99.5%		91.2%	

TABLE II

CORRELATION BETWEEN E_4, E_3, E AND F CONCENTRATIONS MEASURED BY RIA AND HPLC

Sample No.	E_4 (ng/ml)		E_3 (ng/ml)		E (ng/ml)		F (ng/ml)	
	RIA	HPLC	RIA	HPLC	RIA	HPLC	RIA	HPLC
1	0.71	0.40	9.67	10.75	84.1	53.7	371.0	202.5
2	3.53	3.77	56.53	65.45	240.0	208.5	292.0	157.5
3	9.59	5.44	174.96	206.60	102.0	89.7	296.0	188.7
4	1.05	1.86	10.57	4.62	11.2	4.6	57.6	43.8
5	0.76	0.57	18.41	31.39	108.0	79.9	364.0	259.0
6	4.43	4.92	148.71	159.65	265.0	198.1	165.0	118.0
7	4.81	1.87	43.34	45.63	33.4	28.9	142.0	119.7
8	1.49	0.43	19.76	15.80	91.4	53.8	610.0	473.4
9	3.37	4.72	59.63	113.54	164.0	168.7	165.0	118.6
10	4.75	3.17	62.51	77.19	204.0	173.4	209.0	177.9
11	3.33	2.39	62.35	74.56	155.0	143.6	231.0	188.6
12	1.81	1.61	23.97	16.98	6.0	5.5	95.6	78.5
13	0.61	0.20	15.71	15.17	63.4	43.7	458.0	300.9
14	5.74	2.69	155.97	166.39	161.0	132.9	202.0	183.7
15	2.20	0.89	36.95	42.19	167.0	136.9	265.0	166.3
16	0.43	0.22	12.73	14.96	128.0	102.3	236.0	108.5
17	3.97	1.58	142.25	108.46	269.0	204.2	252.0	360.5
18	1.59	1.18	59.54	60.29	147.0	143.2	150.0	127.8
19	1.77	0.44	47.86	46.13	171.0	111.3	155.0	128.9
20	4.50	4.69	63.79	63.53	174.0	197.3	187.0	145.1

TABLE III

CONCENTRATION OF HORMONES IN NORMAL WOMEN AT 37–41 WEEKS OF GESTATION (n = 80)

	E_4 (mean ± S.D.) (ng/ml)	E_3 (mean ± S.D.) (ng/ml)	E (mean ± S.D.) (ng/ml)	F (mean ± S.D.) (ng/ml)
MPV[a]	0.41 ± 1.34	15.6 ± 8.94	58.1 ± 25.0	139.2 ± 90.3
UV	2.01 ± 1.79	102.9 ± 55.5	191.9 ± 282.6	49.3 ± 68.9
UA	1.11 ± 1.07	34.4 ± 30.4	122.8 ± 58.1	58.3 ± 27.7
AF	3.40 ± 4.02	30.1 ± 31.3	13.1 ± 9.83	21.6 ± 30.4

[a] MPV = maternal peripheral vein; UV = umbilical vein; UA = umbilical artery; AF = amniotic fluid.

and 1.7% (n = 5), respectively. Table II compares the values obtained by the present method and RIA (entrusted to Teikoku Zouki Pharmacy Industries, Tokyo, Japan) in twenty samples of maternal peripheral vein (MPV), umbilical vein (UV), umbilical artery (UA) and amniotic fluid (AF) at 36–41 weeks of gestation. There were significant correlations in the values for E_4 ($y = 0.321 + 0.606\,x$, $r = 0.787$), E_3 ($y = 2.643 + 1.050\,x$, $r = 0.957$), E ($y = -1.347 + 0.841\,x$, $r = 0.956$) and F ($y = 17.564 + 0.672\,x$, $r = 0.865$).

Table III shows the mean concentrations of the hormones in MPV, UV, UA and AF at delivery from the normal women at 37—41 weeks of gestation (n = 80). Figs. 2–5 show E_4, E_3, E and F concentrations, respectively, in MPV (n =

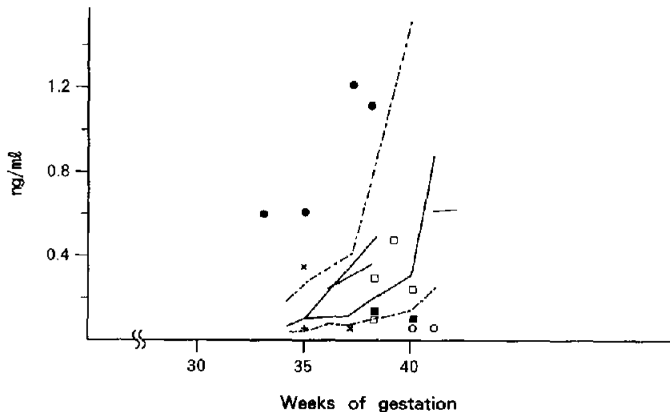

Fig. 2. Concentration of unconjugated estetrol (E_4) in maternal serum as a function of gestation time. Broken lines represent the concentrations of E_4 (mean ± S.D.) in the serum of normal gestational women. Solid lines are the concentrations in serial measurements in the serum of four normal gestational women. (□) Fetal distress; (○) toxemia with fetal distress; (×) toxemia without fetal distress; (●) twins; (■) intrauterine growth retardation (IUGR); (+) intrauterine fetal death (IUFD).

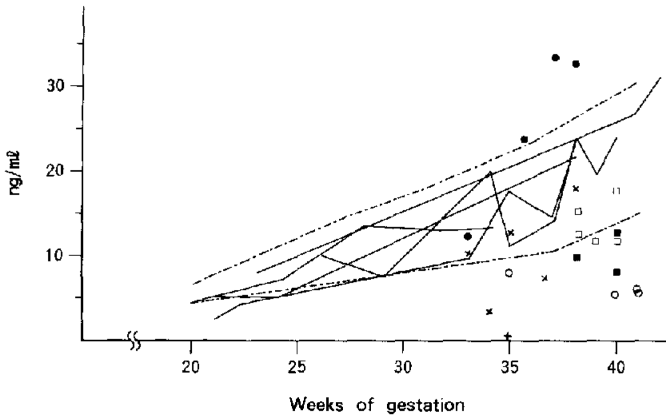

Fig. 3. Concentration of unconjugated estriol (E_3) in maternal serum as a function of gestation time. Broken lines represent the concentrations of E_3 (mean ± S.D.) in the serum of normal gestational women. Solid lines are the concentrations in serial measurements in the serum of five normal gestational women. (□) Fetal distress; (○) fetal distress with toxemia; (×) toxemia without fetal distress; (●) twins; (■) intrauterine growth retardation (IUGR); (+) intrauterine fetal death (IUFD).

180) plotted against gestation time. As shown in Fig. 2, the concentration of E_4 could be determined after 33 weeks of gestation. The E_4 and E_3 concentrations in the MPV of cases complicated by intrauterine growth retardation (IUGR) or intrauterine fetal death (IUFD) had a tendency to be lower than those of the normal cases, as shown in Figs. 2 and 3. The E_4 and E_3 concentrations in cases of

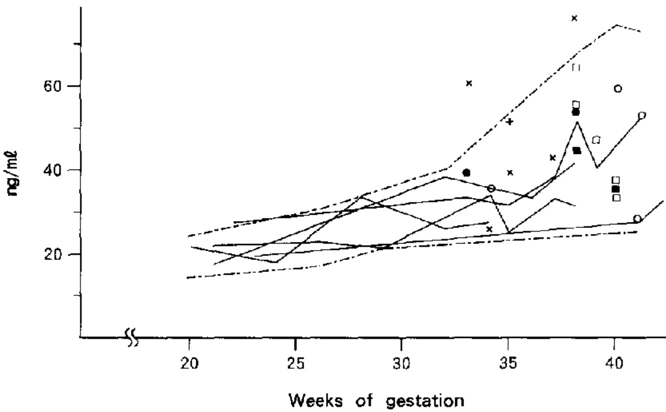

Fig. 4. Concentration of unconjugated cortisone (E) in maternal serum as a function of gestation time. Broken lines represent the concentrations of E (mean ± S.D.) in the serum of normal gestational women. Solid lines are the concentrations in serial measurements in the serum of five normal gestational women. (□) Fetal distress; (○) fetal distress with toxemia; (×) toxemia without fetal distress; (●) twins; (■) intrauterine growth retardation (IUGR); (+) intrauterine fetal death (IUFD).

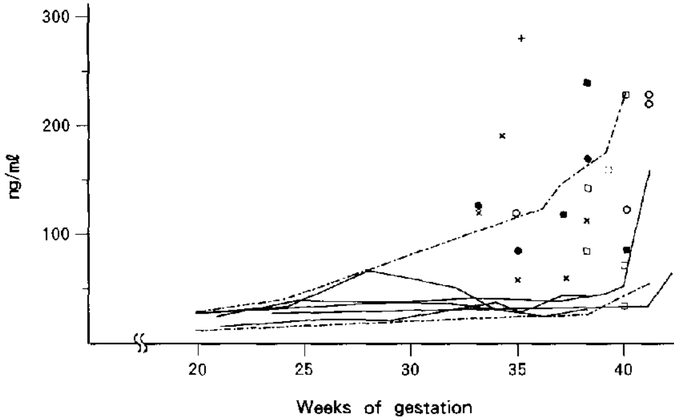

Fig. 5. Concentration of unconjugated cortisol (F) in maternal serum as a function of gestation time. Broken lines represent the concentrations of F (mean ± S.D.) in the serum of normal gestational women. Solid lines are the concentrations in serial measurements in the serum of five normal gestational women. (□) Fetal distress; (○) fetal distress with toxemia; (×) toxemia without fetal distress; (●) twins; (■) intrauterine growth retardation (IUGR); (+) intrauterine fetal death (IUFD).

twins were higher than in normal cases. As far as the toxemia cases with fetal distress on fetal heart-rate monitoring are concerned, the E_4 and E_3 concentrations were lower than those of the normal cases, but the toxemia cases without fetal distress showed no pattern of the E_4 and E_3 concentrations. The E concen-

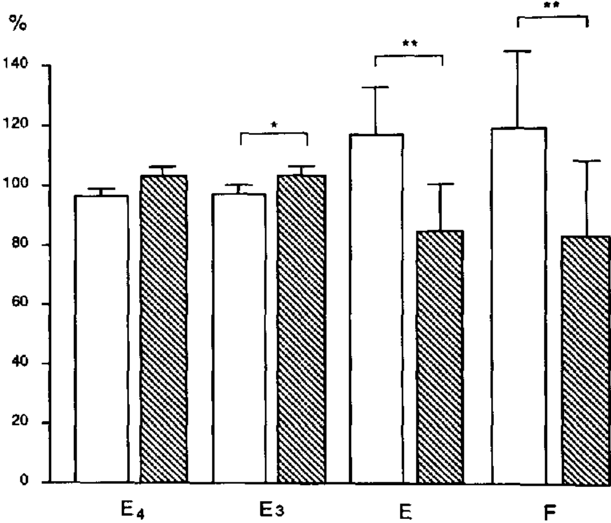

Fig. 6. Diurnal changes of the hormones in maternal serum. Mean serum concentrations of unconjugated estrogens and glucocorticoids at 9 a.m. (□) and 9 p.m. (▨) were assayed in the four normal gestational women at 35–41 weeks. The ratio represents the observed values from the average of the corresponding day. Error bars show the S.D. $* = p < 0.05$; $** = p < 0.025$ ($n = 16$).

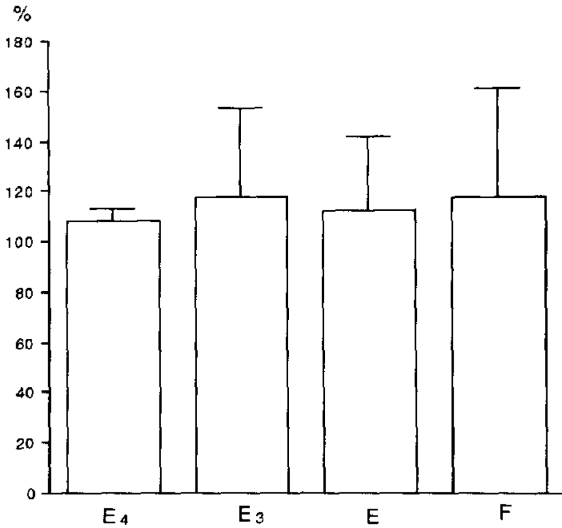

Fig. 7. Day-to-day variation of the hormones in maternal serum. The ratio represents the mean percentage of unconjugated estrogens and glucocorticoids from each preceding day's value. Serum samples were obtained at 9 a.m. from four normal gestational women at 35–41 weeks. The error bars show the S.D. (n = 12).

trations of the normal and the complicated cases exhibited no difference (Fig. 4). Five out of seventeen complicated cases showed high F concentrations (Fig. 5).

Fig. 6 shows the diurnal changes of the hormonal concentrations in MPV. Samples were taken at 9 a.m. and 9 p.m. for 4 days at 35–41 weeks of gestation from four normal women. The mean ratio of the E_4, E_3, E and F concentrations in MPV was calculated from each average value of the corresponding days. There were statistical differences (Wilcoxon test) between 9 a.m. and 9 p.m. for the hormones, except for E_4 (E_3, $p < 0.05$; E, $p < 0.025$; F, $p < 0.025$). The concentrations of estrogens were lower at 9 a.m. than at 9 p.m., whereas the concentrations of glucocorticoids showed the opposite behaviour. In each case, the maximum decreases in E_4, E_3, E and F concentrations were 6.4, 7.8, 36.1 and 51.5%, from each average value of the corresponding day.

Fig. 7 shows day-to-day variations of the hormonal concentrations in the MPV. The ratio represents the mean percentage of E_4, E_3, E and F from each preceding day's value. The mean of the hormonal level showed an increase, but without statistical significance. The E_4 level did not show a decrease in any case. In each case, the maximum decreases in E_3, E and F were 24.5, 21.2 and 22.2%, and the maximum increases in E_4, E_3, E and F were 15.2, 28.6, 85.2 and 87.2%.

DISCUSSION

Hayashi *et al.* [9] reported previously the measurement of E_4, E_3, E_2 and E_1, using HPLC after solvent extraction, evaporation and reconstitution. Hayata *et*

al. [10] reported the assay of E_2, E_1, progesterone, 17α-hydroxyprogesterone and testosterone by the same method. Later, Suzuki *et al.* [8] developed an automated direct assay system. In this study, we modified these methods in the assay of E_4, E_3 and E and F in serum and amniotic fluids, to produce reproducible results with good recoveries and sensitivity. Moreover, this method could be used to measure E_3, E_2 and E_1 simultaneously in serum and amniotic fluids, by changing the proportions of phosphate buffer solution, acetonitrile and methanol in the eluent (10:4:4, v/v).

The E_4 levels of MPV in this study were similar to those reported by Tulchinsky *et al.* [1] and Sagara *et al.* [11], and lower than those by Bennassi *et al.* [12]. As for the E_4 levels of UV, UA and AF, our data were similar to those obtained by HPLC, but much lower than those obtained by RIA. In this study, the E_4 level in UV was statistically higher than that in UA ($p < 0.01$). Takagi *et al.* [13] and Sagara *et al.* [11] also reported that E_4 in UV was higher than in UA, but without statistical significance.

E_4 is thought to be formed via two main pathways, the phenolic pathway, in which estrogens are finally 15-hydroxylated, and the neutral pathway, in which C_{19} steroids are first 15-hydroxylated in the fetus and then aromatized in the placenta [14,15]. Thus the results of this study might indicate that the neutral pathway is more important. The E_3 levels in serum and amniotic fluid were similar to those obtained by RIA [16,17].

The twin cases had high E_4 and E_3 concentrations in the MPV, and the IUGR cases had low values. This means that E_4 and E_3 levels in the MPV are directly related to the feto-placental function. The toxemia cases with fetal distress showed low E_4 and E_3 concentrations. The toxemia cases without fetal distress showed various E_4 and E_3 concentrations, which may indicate that each toxemia case had different pathogenesis and states.

The F concentrations in serum or amniotic fluids were similar to those reported by Murphy [18] and Turner *et al.* [19]. In this study, some of the complicated cases exhibited high F concentrations in the MPV. As to the mode of delivery, the F concentrations in the MPV of the vaginal delivery cases were higher than the elective caesarean section cases. Thus F levels in the MPV may indicate the stress intensity of the mother, other than that of the fetus or placenta. The E concentrations in the MPV of complicated cases were not different from those of uncomplicated cases.

As E_3 levels in the MPV are influenced by daily variations of E or F levels, monitoring of the feto-placental function must include analysis of the profile of hormone levels.

This system enables the simultaneous assay of the hormones, and offers the rapidity and reproducibility needed for clinical monitoring of feto-placental function and maternal state.

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