caseosa) which could change completely the end results of fatty acids analysis. The recognition that centrifugation at 3.500 x g at room temperature can be substitued for centrifugation at 20.000 x g for 40 minutes at low temperature ($<5°C$) as originally suggested ([3]) further increases the availability of the present technique.

SUMMARY

A simplified method for gas-chromatographic determination of fatty acids in amniotic fluid is described. The present results on a series of normal and pathological cases further support the conclusions of other investigations that in amniotic fluid a palmitic acid/stearic acid ratio equal or greater than 5 is reliable indicator of attained fetal lung maturity.

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Circadian rhythm of plasma oestriol and urinary oestriol in pregnancy at term

by

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The use of plasma hormonal analyses as a means for establishing the functioning and condition of the foeto-placental unit is now widespread.

Among the various hormones of foeto-placental origin whose use has been suggested for the monitoring of pregnancy, the most reliable nowadays seems to be oestriol. Its levels in the plasma and urine behave in such a way as to provide a sufficiently safe and effective index within the scope of a surveillance programme, as complete as possible, in the last weeks of pregnancy ([1]), also because

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the radio-immunological and gas chromatographic methods used today enable rapid and precise analyses to be made in both the blood and the urine.

During pregnancy the synthesis of oestriol by the placenta is linked to the availability of both maternal and foetal precursors. Changes in the production of these precursors are necessarily reflected in corresponding modifications of oestrogenic synthesis in general and of oestriol in particular. Since there is a circadian rhythm in the production of these cortical precursors, it has been suggested that a similar rhythm could be found also in oestrogen synthesis ([2,3]).

Both theoretical and practical considerations, e.g., the fact that the mean value is not necessarily very close to that of the individual determination or to that of the initial or final determination, demonstrate the importance of the discovery of a daily oestriol rhythm in the blood and urine, both for the purposes of better understanding of the control mechanisms of the oestrogen synthesis processes, and to provide a more exact interpretation of the modifications as verified by the levels of this hormone when the situation of the foeto-placental unit is assessed.

In six pregnant women at term, we examined the variations in the plasma and urinary levels of oestriol during 24 hours; we also attempted to elucidate the existence of possible correlations between the behaviour of the plasma oestriol and the nature of its urinary excretion.

MATERIAL AND METHODS

Our trial was carried out on 6 pregnat women admitted to the Obstetric and Gynaecological Clinic of the University of Genova. They were between 20 and 30 years of age (mean age 24) four of them were primiparae and two multiparae.

The age of pregnancy varied from 38 to 41 weeks. None of the subjects under consideration presented with any complications during the course of pregnancy; five subsequently gave birth by the vaginal route, one by the abdominal route because of a presentation anomaly; all the neonates were in good health.

Because of the brief half-life of oestriol, samples were obtained every 3 hours for 24 hours, beginning at 10 a.m. The sample, once obtained, was immediately centrifuged and the plasma was preserved at −15°C until analysed.

Urine was collected from the same subjects every 6 hours for 24 hours, likewise starting at 10 a.m. The urines were measured in each case and a sample was obtained for the analysis of oestriol and creatinine.

For the radio-immunological analysis of oestriol in the blood we used Sorin's kit. This kit is based on the following procedure: 1) extraction of oestriol from the plasma or the serum; 2) incubation of the reaction mixtures for 30 minutes at 37°C for 2 hours at 4°C; 3) absorption of the free oestriol on charcoal-dextran; 4) centrifugation at room temperature and calculation of the supernatant fluid.

Each kit contains two series of reagents, made up of: a) tritiated oestriol; b) standard oestriol; c) anti-oestriol antiserum; d) tris buffer 0.025 M, pH 7.4, 0.25% lysozyme; e) charcoal-dextran, dry mixture.

The extraction of oestriol from the sample of plasma was done by adding ethyl ether. The standard solution containing 4 ng/ml was gradually diluted with tris buffer so as to obtain solutions at concentrations suitable for the 0.125-4 ng/ml interval.

In calculating the results, the basic mean for counting each group of test-tubes was first determinated, then the binding capacity of the system of analysis was

calculated as percentage ratio between the mean relating to the « total activity ». The binding capacity was normally between 30 and 50%. The mean of the counts related to each standard and the mean of the samples being tested was expressed as a percentage in relation to the mean referring to the « standard zero ».

$$(B/B_0)\% = \frac{\text{Standard or samples mean count}}{\text{« Zero standard » mean count}} \times 100$$

The percentages calculated for the standards were plotted on a linear graph in terms of the oestriol content. The calibration curve was thus constructed. Then the content of oestriol in each sample being tested was read directly on the calibration curve. If necessary the results were corrected for the extraction yield, dividing it by the yield factor YF ([4]) and brought to normal at 1 ml based on the volume of plasma extracted and the quantity of extract utilized.

The urinary oestriol was analysed by Brombacher's fluorimetric method; the urinary creatinine was analysed by a colorimetric method (Farb-Test, Biochemica).

All the analyses, both in the blood and the urine, were done in duplicate. The coefficient of variation of the duplicates, determined on the first 24 samples examined, was 6.8%. Any determinations in which there were differences in the duplicates in relation to the mean value of more than ±9.7% were considered unreliable and were therefore repeated.

The values obtained were worked out by means of an « intertechnique Multi 4 » computer.

RESULTS

The values for plasma oestriol found in the six patients examined during the period of observation are given in Table I. The individual level at 1 p.m. varied, on average, by a minimum of 11.76% and a maximum of 41.37% as compared to those of 4 p.m. The mean maximum value over 24 hours, obtained during the day at 1 p.m., was significantly higher than the mean minimum value at 24 hours, obtained during the night at 4 a.m. ($p < 0.025$).

The analysis of variance for the plasma oestriol values was not statistically significant either within the groups or between them.

Tab. 1. *Daily variations in plasma oestriol (ng/ml) in six pregnant women at term.*

Hour	10 a_1	13 2_2	16 a_3	19 a_4	22 a_5	1 a_6	4 a_7	7 a_8	10 a_9	Σ $\bar{a}$
T.G.	16	16.4	15.6	14.6	15.6	12.6	11.6	13.2	14.8	14.48
L.R.	13.4	12.2	11.6	13.2	7.4	11.8	10	8.6	11.8	11.11
S.C.	11.2	7.8	8.4	5.8	5.5	7	6.8	6.2	12.1	7.86
P.E.	6.07	10.07	9.4	10.3	10.8	8.03	8.4	9.8	5.9	8.75
P.L.	12.6	9.5	8.9	9.5	11.4	9.4	8.5	13.4	11	10.46
F.A.	5.3	9.6	6.6	5.4	5.8	7	7.5	6.4	5.8	6.59
M	10.76	10.92	10.08	9.76	9.41	9.30	8.79	9.60	10.23	9.87
D.S.	4.23	3.02	3.14	3.74	3.91	2.42	1.74	2.95	3.62	
E.S.	1.73	1.23	1.28	1.53	1.59	0.98	0.71	1.11	1.48	

M=mean value; D.S.=standard deviation; E.S.=standard error.
a=mean individual daily value of plasma oestriol.

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Tab. 2. *Daily variations in oestriol/creatinine ratio in six pregnant women at term.*

Hour	10-16 E/C_1	16-22 E/C_2	22-4 E/C_3	4-10 E/C_4	
T.G.	33.1	22	17.8	21.6	23.62
L.R.	22	19	16	17.1	18.52
S.C.	17.9	16.6	27.7	14.9	19.27
P.E.	65.4	43.4	28.1	30.2	41.77
P.L.	22	23.5	19.9	21	21.60
F.A.	32.3	30.7	19.8	29	27.95
M.	32.11	25.85	21.55	22.30	25.45

M=mean value; E/C=oestriol/creatinine ratio.
E/C=mean individual daily value of oestriol/creatinine ratio.

The individual concentrations at 10 a.m., i.e. when the observations started, were similar to those recorded at 10 a.m. on the following day, i.e. at the end of the trial, in all the subjects considered.

The values for the oestriol/creatinine ratio in the urines of the individual fractions considered for each subject during the 24 hours are shown in Table II, together with the total mean value of the oestriol/creatinine ratio in the 24-hour urines of each subject, and finally the mean values for the same ratio in the fractions considered individually for the subjects taken in aggregate ([5,6]).

The analysis of variance between the mean values for the oestriol/creatinine ratio of the individual fractions during 24 hours, calculated from the individual values expressed as a percentage of the daily mean for each subject, shows the existence of daily variations that are statistically significant ($p<0.05$).

Fig. 1 shows the percentage differences between the oestriol/creatinine ratio of each fraction and that of the 24-hour sample.

Obvious fluctuations were found in the values both for plasma oestriol and

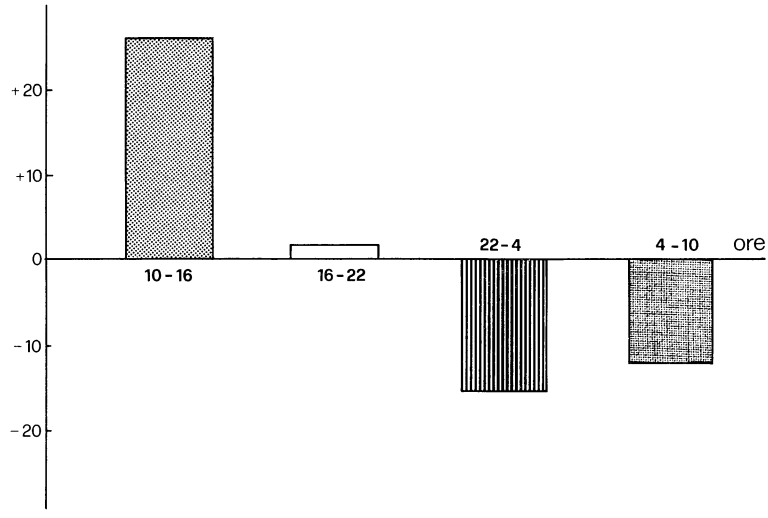

FIG. 1 - Daily variations in oestriol/creatinine ratio expressed as a percentage of mean value of all the subjects in 24 hours.

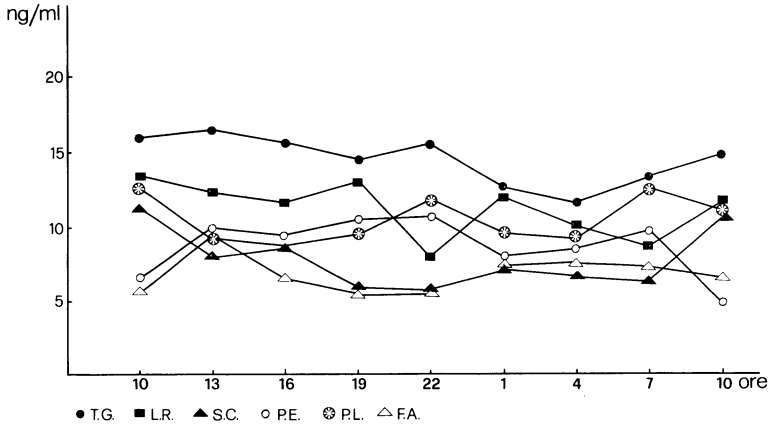

FIG. 2 - Daily variations in plasma oestriol in 24 hours in the six subjects considered.

the oestriol/creatinine ratio in all the subjects. The changes in plasma oestriol during 24 hours in each patient examined are shown in Fig. 2.

In order to find out what intervals of time gave rise to the variations encountered, both for the plasma values of oestriol and for the oestriol/creatinine ratio in the urine, a comparison of the means was made by Neumann-Keuls' method ([7]). We found that the means recorded at 10 a.m. for plasma oestriol showed a statistically significant difference from those recorded between 10 p.m. and 1 a.m. ($p < 0.02$); the mean values for the oestriol/creatinine ratio at 12 noon differed significantly from those at 12 midnight ($p < 0.01$).

In order to assess the existence or otherwise of a correlation between the plasma levels of oestriol and the nature of its urinary excretion, even when expressed as an oestriol/creatinine ratio, we compared the mean values of oestriol in the blood at intervals of time corresponding to the collection of the individual fractions of urine with the values for the oestriol/creatinine ratio in the same urinary fractions.

The coefficient of correlation was 0.98 with levels of significance at 5% of 0.95 and at 1% of 0.99.

This correlation is clearly expressed in Fig. 3, which represents the graphic expression of the square equation found from a comparison with the coefficient of correlation, the levels of significance and the coefficient of regression. The graph clearly shows how the two variables compared (plasma oestriol and the oestriol/urinary creatinine ratio), tend to vary conjointly.

CONCLUSIONS

While numerous studies have been made that demonstrate daily variations in the urinary excretion of the oestrogens in pregnancy at term ([8, 9, 10]), only a few data have so far been published on their plasma levels and on variations of them during the course of 24 hours in analagous circumstances.

Munson *et al.*, ([11]) have reported mean plasma concentrations of 17-beta-oestradiol that were significantly lower at 9 pm. than at 8 a.m. in 11 pregnat women at term; Selinger & Levitz ([1]) reported that in 18 subjects out of 25 that were

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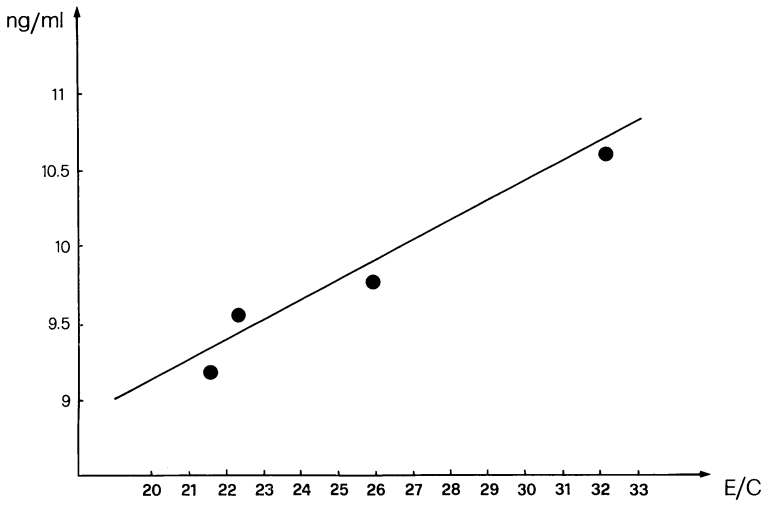

FIG. 3 - Correlation between plasma oestriol and oestriol/creatinine ratio in 24 hours, expressed by square equation.

considered, the plasma levels of oestradiol, measured for two consecutive days, were significantly lower at 4 p.m. that at 8 a.m. The data supplied by Townsley *et al.* ([9]) on the total plasma oestriol during 24 hours showed that there was a daily rhythm rather than a day-night rhythm. Tulchinsky *et al.* ([12]) reported significantly higher values at midnight than at 8 a.m. ($p < 0.05$) for nonconjugated plasma oestriol. Finally Goebel & Kuss ([10]), in a recent study, confirmed the existence of a circadian rhythm for non-conjugated plasma oestriol, with maximum values at 8 p.m. and minimal at 8 a.m.

The data supplied by the various authors cited differ from our own results. In fact, our trial demonstrated the existence of a circadian rhythm for plasma oestriol with the highest values at 1 p.m. and the lowest at 4 p.m. (Fig. 4). This

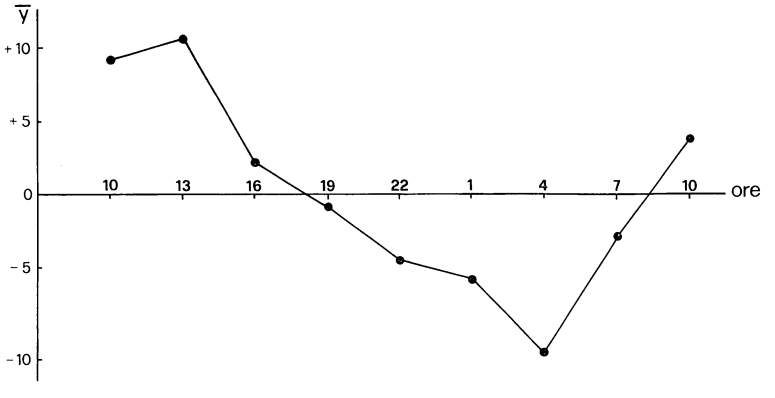

FIG. 4 - Daily variations in plasma oestriol, expressed as percentages of the mean values of all the subjects in 24 hours.

rhythm was evident despite the marked variations in hormonal values that may be encountered in the individual times considered, in the subjects examined.

It seems to us that the ratio existing between the plasma values of oestriol and the levels of its urinary excretion, well shown by application of the square equation, is particularly interesting. The clear correlation existing between the two variables considered on the one hand provides justification for using either plasmatic or urinary oestriol as a test of foeto-placental function, and on the other constitues an indirect confirmation of the exactness of the values obtained by us with the methods used.

Various and complex factors compete in the determination of plasma values of oestriol, involved either in the synthesis (from foetal and maternal precursors) or in the metabolism and renal clearance of this hormone. It is clear that in order to explain the daily variations encountered in plasmatic and urinary oestriol we need to bring up modifications occurring in these processes.

The scarcity of our knowledge concerning the inner and delicate mechanisms of the complex phenomena involved in these processes makes it impossible to identify with any certainty what these differences are, or at what levels of synthesis, metabolism or excretion they act. It seems more probable, however, that variations in the maternal cortical function bear a greater share of the responsibility than do the foetal variations ([9]). The fact that the daily variations are small as compared with the mean levels, as might be expected if the rhythm depend on the smallest of the various sources of precursors, would be in accordance with this hypothesis, and in fact neither the maternal nor any other circadian rhythm has been identified for the foetal cortex, which, since it can respond normally to ACTH, does not present, up to the 2nd or 3rd day of life, any ciclic progress of the production of corticoid hormones of the adult type, which implies the absence of any rhythmic activity during foetal life.

Whatever may be the factor responsible for this circadian rhythm, it seems that it requires the necessity for standardization of the times of sampling, so as to minimize the influence of the rhythmic variations upon the hormonal values found, thus adding to the validity of the method of determining oestriol in the blood and urine, as an index of the state of the foeto-placental index within the context of precise, rapid and safe monitoring of pregnancy at term.

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