Technical methods

A simple radioimmunoassay for serum β -oestriol in pregnancy

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Most clinical laboratories offer determination of the 'total oestrogen' output on a timed urine specimen as a test of fetal function. These assays can conveniently be automated. However, they have a number of disadvantages, namely, the inconvenience of collection timing, the 12- or 24-hour delay, uncertainties due to failure to empty the bladder, and occasional interference from drug metabolites and glucose. An alternative approach is to measure the plasma oestriol level, and an original radioimmunoassay method which is fast enough to allow the reporting of results on the same day has now been developed. The results with this procedure suggest that it may with advantage be substituted for the urine 'oestriol' assay in the assessment of fetal function.

Method

ANTISERUM

Antisera were raised in rabbits to conjugates of oestriol-6-o-carboxymethyl oxime and various mammalian albumins. A conjugate of rabbit albumin produced high-titre antibody with high specificity for oestriol. This antiserum (1.0 ml) was used at a dilution of 1 in 10 000 in carbonate/bicarbonate buffer (0.05 M, pH 9.6) to coat polystyrene tubes (Sarstedt 10 $\times$ 55 mm) with a minimum contact time of 24 hours at 4°C according to the method of Catt and Tregear (1967).

When required for the assay, each tube is aspirated and treated twice with 1 ml phosphate buffered saline (0.04 mol/l phosphate, 0.6% NaCl pH 7.4) then with 1.0 ml of a similar phosphate buffered saline but containing 0.1% bovine albumin.

TRACER

The tracer is prepared from oestriol-6-o-carboxymethyl oxime BSA (Steraloids, Croydon) and ^{125}I sodium (Radiochemical Centre, Amersham) by the

method of Hunter and Greenwood (1962) and the iodination mixture is fractionated on Sephadex G-15 and eluted with buffer containing 1% bovine albumin. The pooled fractions containing the ^{125}I steroid-albumin conjugate are stored at 4°C. In the assay the tracer solution is diluted in phosphate buffered saline so that 100 μl contains the equivalent of approximately 50 pg of steroid (about 1/70 dilution), giving 5-10 000 counts/minute bound in the zero tube.

HYDROLYSIS

Hydrolysis, prior to assaying, is carried out as follows. Glass tubes (100 $\times$ 18 mm) are set up, each containing 200 μl water plus 10 μl serum from a bank of control serum or from patients' blood. Standards comprise 10 μl pooled male serum together with 200 μl serially diluted oestriol in phosphate buffered saline to cover the range 5, 2.5, 1.2, 0.6, and 0.3 ng/tube. After 200 μl HCl (5 mol/l) has been added the tubes are loosely stoppered with plastic bubbles, heated in a boiling waterbath for 20 minutes, rapidly cooled in running water, and made alkaline with 1.0 ml NaOH (1 mol/l).

RADIOIMMUNOASSAY

Each hydrolysate (50 μl) is transferred to the antibody-coated tubes containing 1.0 ml phosphate buffered saline plus bovine albumin. After 1 hour at 4-8°C 100 μl tracer is added and after a further 3-4 hours at room temperature the tubes are aspirated, washed once with water, and counted in an Auto-gamma (LKB Wallac 8000) counter, accumulating at least 10 000 counts in the zero tube.

Urine oestrogens (calculated as oestriol) were determined by the method of Oakey *et al* (1967) modified by the use of 1 ml urine and incorporating a water wash following the bicarbonate wash. In routine practice, over several years the National Quality Control Survey showed a precision of between 3 and 4 mg/day.

CALCULATION

A graph is constructed with counts (or percentage bound) on the vertical axis and the concentration of the standard on the horizontal axis. Extrapolation of the mean serum count from this standard curve yields the oestriol value.

RELIABILITY OF TECHNIQUE

The within-batch CV for 20 assays was found to be

Received for publication 6 January 1976

3.8%. The assay CV for between-batch variations was 8.6%; eg, one control serum assayed daily gave 0.46, 0.38, 0.39, 0.37, 0.46, 0.46, 0.42, 0.38, 0.43, 0.48, 0.39, 0.41 $\mu\text{mol/l}$. Varying degrees of haemolysis (up to 10 g/l) were demonstrated to have no effect on the assay results. The mean and range of 'normal' serum total oestriol values late in the third trimester are shown in table I. These agree with recent findings using a tritiated label in this laboratory (Stafford, 1973) and elsewhere (Goebelsmann *et al*, 1975).

Gestation (weeks)	Women (no.)	Oestriol ($\mu\text{mol/l}$)	
		Range	Mean
32-33	38	0.16-0.30	0.23
34-35	22 (23 ¹)	0.20-0.34 (0.20-1.7) ¹	0.25 (0.40) ¹
36-37	38	0.23-0.73	0.50
38-39	50	0.26-1.5	0.82
40	38	0.21-1.7	0.82

Table I Serum total oestriol concentration in 27 healthy pregnant women under conditions of unrestricted activity

¹Includes one twin pregnancy.

SPECIFICITY

A check for the antiserum specificity showed there was negligible cross-reaction with oestradiol, oestrone, 17-epioestriol, 16, 17-epioestriol, cortisol, pregnanediol, progesterone, dehydroepiandrosterone, and 16 α -hydroxyoestrone. Cross-reaction between the antiserum and 6-oxo-oestriol, oestrol, and 16-epioestriol occurred to the extent of 147, 67, and 100% respectively. This is

Figure Circadian rhythm in serum total oestriol throughout 36 hours in late pregnancy. Ordinate: actual percentage deviation from the mean of six individual values. Abscissa: time of day or night in hours.

irrelevant because of the very much lower levels of these substances in pregnancy blood (Adlercreutz and Luukkainen, 1970; Katagiri *et al*, 1974; Tulchinsky *et al*, 1975).

CIRCADIAN FLUCTUATION

The periodicity of the oestriol concentration in the blood serum is shown in a composite graph (figure); low levels in the early morning rise during the afternoon and evening before dropping again.

Patient	Gestation (weeks)	Condition	Urine Oestrogens			Serum Oestriol	
			Day (mg)	Night (mg)	24 hours (mg)	ng/ml	$\mu\text{mol/l}$
TV	36	Anaemia, IU growth retardation	11	6	17	77	0.27
SF	36	Pre-eclampsia, abnormal GTT	10	6	16	126	0.44
ER	36	Pre-eclampsia	9	9	18	89	0.31
VB	37	Pre-eclamptic toxemia	12	7	19	201	0.70
BS	38	Twin pregnancy	—	—	37	246	0.85
SS	38	Previous stillbirth	7	7	14	153	0.53
CD	39	Unstable lie	—	—	18	270	0.94
LW	39	Epileptic. Abnormal GTT	18	10	29	432	1.50
GL	39	Reduced fetal activity	10	14	24	281	0.97
LU	41	Hypertension. Reduced fetal activity	8	7	15	185	0.64
TD	40	Pre-eclamptic toxemia	5	8	13	134	0.46
AB	40	Unstable lie, gravida 5	16	15	32	500	1.7
JD	40	Hypertension at term	14	18	32	361	1.25

Table II Total oestriol in the blood serum and day time urine (08.00-20.00) and night urine (20.00-08.00) of 13 hospitalized pregnant women of 36-40 weeks' gestation

GTT = glucose tolerance test IU = intrauterine

Patient	Urine				Serum			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
SS	+ 14	+ 43	- 14	- 36	- 13	+ 2	+ 2	+ 8
SR	- 33	- 19	+ 26	+ 26	- 6	- 9	+ 14	+ 3
FH	- 35	- 35	+ 48	+ 30	- 12	- 38	+ 9	+ 41
TV	0	- 12	+ 18	—	0	+ 5	- 4	—
VB	- 26	- 21	+ 32	+ 16	- 4	+ 17	- 3	- 10
SF	- 19	- 8	+ 30	—	+ 27	- 3	- 12	- 11
TD	+ 8	+ 23	+ 8	- 31	- 18	- 16	+ 15	+ 21
JD	- 22	+ 3	- 8	+ 25	- 24	+ 1	+ 11	+ 12
TB	+ 18	- 9	+ 9	- 9	- 7	+ 9	- 10	+ 8
ER	0	0	+ 11	- 22	- 15	+ 16	- 10	+ 9
GL	- 8	+ 13	+ 18	- 23	+ 19	- 1	- 30	+ 14
CM	+ 55	- 4	- 41	- 11	- 37	0	+ 10	+ 28

Table III Percentage mean variations in serum and 24 h urine oestriol values in 12 hospitalized women

COMPARISON WITH URINE ASSAYS

Table II shows the mean day and night 'oestriol' excretion and serum oestriol levels from 13 hospitalized 'at risk' pregnant women. Three patients showed a greater night than day 'oestriol' output, four patients excreted more 'oestriol' during the day; no appreciable difference in night and day 'oestriol' output occurred in another four patients. A wide range of urine and serum oestriol values was recorded.

The extent of variation between the individual oestriol results of four consecutive daily urine and blood sera from 12 patients is shown in table III. No overall reductions in urine 'oestriol' variation were achieved by expressing the results as mg/g or $\mu\text{mol/mol}$ creatinine. Some relevant laboratory findings on four cases of intrauterine anoxia are given in table IV.

Gestation and Delivery	Consecutive 24 h Urine 'Oestriol' (mg)	Serum Oestriol (nmol/l)	Serum h-PL (mg/l)
34 weeks, stillborn	6, 4, 2	< 0.03	—
37 weeks, stillborn	1, 1	0.02, 0.02	4.6, 4.6
38 weeks, RDS; died 8 h after birth	8, 9	0.10	—
35 weeks, stillborn	4, 1	0.03	3.4
Normal: 36-40 weeks	> 8	> 0.20	> 4.0

Table IV Urine oestrogens, serum oestriol, and h-PL in four cases of fetal dysfunction

RDS = respiratory distress syndrome

Discussion

A satisfactory oestriol radioimmunoassay for obstetric service demands an oestriol antiserum of high specificity which both increases the accuracy and obviates the need for a solvent or chromato-

graphic partition step. The present method fulfils these requirements. Furthermore, because of the availability of inexpensive automatic gamma counters, the replacement of the tritium label used in currently available oestriol techniques by a gamma tracer brings, for the first time, the RIA within the scope of the small clinical laboratory.

Because of a regular plasma oestriol rhythm at least in some women, repeat blood samples should be taken at about the same time of the day. Even so, the constancy of the daily serum oestriol is only a little better than the urine oestrogen output (table III), daily differences of which arise from spasmodic changes in renal clearance (Dickey *et al*, 1966). Earlier studies on plasma oestriol (Macourt *et al*, 1971; Masson and Wilson, 1972; Townsley *et al*, 1973), which were confined to a testing period of 24 hours, revealed no significant diurnal variation; Townsley *et al* (1973) reported an unconvincing mean diurnal fluctuation of about $\pm 10\%$, the lowest levels being at 20.00 hours and the highest at 12 noon. This is not in agreement with our findings. In each of seven women tested four-hourly for 36 hours, the serum total oestriol level was low in the morning and rose in the afternoon and evening. These contrasting findings may be a reflection of the increased specificity of the present RIA over the fluorimetric method used by Townsley *et al* (1973). Certainly, the circadian rhythm reported here closely mirrors that recently found by Goebel and Kuss (1974) for serum unconjugated oestriol.

We are indebted to the volunteers, the patients, and the obstetric departmental staff at Upton Hospital, Slough and at Heatherwood Hospital, Ascot for their help in evaluating the technique. We thank Mrs K. Pocock for carrying out some of the assays. This work is supported by a research grant from the Oxford Regional Health Authority.

References

- Adlercreutz, H. and Luukkainen, T. (1970). Identification and determination of oestrogens in various biological materials in pregnancy. *Ann. clin. Res.*, **2**, 365-380.
- Catt, K. J. and Tregear, G. W. (1967). Solid-phase radioimmunoassay in antibody-coated tubes. *Science*, **158**, 1570-1572.
- Dickey, R. P., Carter, W. T., Besch, P. K., and Ullery, J. C. (1966). Effect of posture on estrogen excretion during pregnancy. *Amer. J. Obstet. Gynec.*, **96**, 127-130.
- Goebel, R. and Kuss, E. (1974). Circadian rhythm of serum unconjugated estriol in late pregnancy. *J. clin. Endocr.*, **39**, 969-972.
- Goebelsmann, U., Katagiri, H., Stanczyk, F. Z., Cetrulo, C. L., and Freeman, R. K. (1975). Estriol assays in obstetrics. *J. Steroid. Biochem.*, **6**, 703-709.
- Hunter, W. M. and Greenwood, F. C. (1962). Preparation of iodine-131 labelled growth hormone of high specific activity. *Nature (Lond.)*, **194**, 495-496.
- Katagiri, H., Stanczyk, F. Z., and Goebelsmann, U. (1974). Estriol in pregnancy. III. Development, comparison and use of specific antisera for rapid RIA of unconjugated estriol in pregnancy plasma. *Steroids*, **24**, 225-238.
- Macourt, D., Corker, C. S., and Naftolin, F. (1971). Plasma oestriol in pregnancy. *J. Obstet. Gynaec. Brit. Cwlth.*, **78**, 335-340.
- Masson, G. M. and Wilson, G. R. (1972). Variability of total plasma oestriol in late human pregnancy. *J. Endocr.*, **54**, 245-250.
- Oakey, R. E., Bradshaw, L. R. A., Eccles, S. S., Stitch, S. R., and Heys, R. F. (1967). The rapid estimation of oestrogen in pregnancy to monitor foetal risk. *Clin. chim. Acta*, **15**, 35-45.
- Stafford, J. E. H. (1973). A versatile radioimmunoassay for the determination of serum oestriol in pregnancy. *Ann. clin. Biochem.*, **10**, 159-166.
- Townslay, J. D., Dubin, N. H., Grannis, G. F., Gartman, L. J., and Crystle, C. D. (1973). Circadian rhythms of serum and urinary estrogens in pregnancy. *J. clin. Endocr.*, **36**, 289-295.
- Tulchinsky, D., Frigoletto, F. D., Jr., Ryan, K. J., and Fishman, J. (1975). Plasma as an index of fetal well-being. *J. clin. Endocr.*, **40**, 560-567.

Morphological methods in the study of the gut immune system in man

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The gut associated lymphoid system is arguably the most important part of the body's peripheral immune mechanism.

Almost exclusively only indirect methods have been used in evaluating its status. There is a massive literature concerning the circulating antibody formation to various food, bacterial, and even epithelial antigens in association with a variety of gastrointestinal diseases, and similar studies have also been made on the antibodies secreted into the gut lumen. The peripheral blood lymphocyte function has also been investigated. Transformation to blastoid forms in response to a variety of stimuli and their cytotoxicity for elements of the gut epithelium have been studied intensively. Much of the information produced by these studies has been largely unhelpful

in increasing our understanding of gastrointestinal disease and some has been contradictory.

Little attention has been given to those aspects of the morphological features of the lymphoid system which might affect function but Cottier *et al* (1972), for example, have shown that the histological appearance of a lymph node can often give information on immunological function. Using similar principles Skinner and Whitehead (1974) examined the mesenteric nodes in Crohn's disease and ulcerative colitis. However, of potentially greater importance is the application of immunohistochemical methods with which the exact secretory function of gut immunocytes can be defined in terms of antibody production and immunoglobulin type. Using morphometric methods the number of immunocytes can be accurately counted and their changes monitored on sequential studies. The present progress in the latter type of methodology is the subject of this report.

Methods for Identification of Cell Type

From the above it is clear that the normal morphological parameters have yet to be defined and much has still to be done in this field. Abnormalities are best shown by objective techniques, for example the presence or absence of a single feature, but when changes involve similar cells or tissues to a different

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