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THE RADIOIMMUNOASSAY OF PREGNANETRIOL 3α-GLUCURONIDE

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Summary—The hapten (5β-pregnane-17,20α-diol-3α-yl glucuronide) required to develop the radioimmunoassay was synthesized by an unambiguous chemical synthesis. An immunogenic complex was synthesized by coupling bovine serum albumin (BSA) to the glucuronide by mixed acid anhydride reaction. The immunogenic complex was used to induce the formation of specific antibodies in rabbits. In addition, the required radioligand [6,7-^{3}H]5β-pregnanetriol 3α-glucuronide was prepared with appropriate specific radioactivity. The characteristics of the antibody were determined with regard to specificity and sensitivity and the precision and accuracy of the assay method established. As applied to urine samples this method is superior to existing procedures in that they avoid the hydrolysis step and can be used directly on diluted urine. Examples have been given of useful application of this technique in clinical practice.

INTRODUCTION

Rationale

The biogenesis of cortisol from progesterone which takes place mainly in the adrenal cortex, involves the action of three steroid hydroxylase enzymes. (a) 17α-hydroxylase (EC 1.14.99.9) converts progesterone to 17α-hydroxyprogesterone; (b) 21-hydroxylase (EC 1.14.99.10) converts 17α-hydroxyprogesterone to 17α,21-dihydroxyprogesterone and finally, (c) 11β-hydroxylase (EC 1.14.15.4) converts 17α,21-dihydroxyprogesterone to cortisol.

This biosynthetic pathway is initiated and sustained by corticotropin secretion from the anterior pituitary and it is the circulating concentration of cortisol which regulates this secretion. If the formation of cortisol is impeded as a result of a deficiency or inhibition of any of these hydroxylases, cortisol concentration in the blood falls and corticotropin secretion increases stimulating the formation of excessive amounts of the intermediate compounds. Thus inhibition of the 21-hydroxylase causes an accumulation of 17α-hydroxyprogesterone, which is excreted as pregnanetriol 3α-glucuronide in the urine. Failure of the 11β-hydroxylase leads to the accumulation of 17α,21-dihydroxyprogesterone in the blood and the appearance of large amounts of tetrahydro-compound S (THS) (3α,17,21-trihydroxy-5β-pregnan-20-one) as a 3α-glucuronide in the urine.

Congenital adrenal hyperplasia

A variety of congenital enzymatic defects in the synthesis of cortisol have now been recognized. The deficiency of cortisol leads to an increased release of corticotropin with the result that adrenals become hyperplastic and metabolism is deviated towards the production of androgens. In girl infants virilism appears as pseudo-hermaphroditism and in boys sexual precocity occurs. Hence prompt diagnosis is desirable as most of the harmful effects of the aberrant steroid metabolism can be avoided by early cortisol replacement therapy [1].

Deficiency of C-21-hydroxylase

The commonest form of congenital adrenal hyperplasia is due to a block in hydroxylation at C-21. An accumulation of the intermediate compound 17α-hydroxyprogesterone occurs, with a considerable increase in its urinary metabolites, pregnanetriol and 17-hydroxypregnanolone. The pregnanetriol is excreted in the urine as the conjugated form, pregnanetriol 3α-glucuronide [2, 3].

The object of the present work was to devise a simple radioimmunoassay for urinary 5β-pregnane-17,20α-diol-3α-yl glucuronide to expedite the early diagnosis of 21-hydroxylase deficient form of congenital adrenal hyperplasia and to measure the excretion of pregnanetriol 3α-glucuronide in urine as a means of assessing therapy.

EXPERIMENTAL

Materials and methods

General purpose solvents, except ethanol (R.R. grade; James Burrough, London SE11, U.K.) were obtained from BDH, Poole, Dorset, U.K., and were distilled before use. Bulk steroids were purchased from Diosynth, Morden, Surrey, SM4 5DZ, U.K. Isobutyl chloroformate and tributylamine were sup-

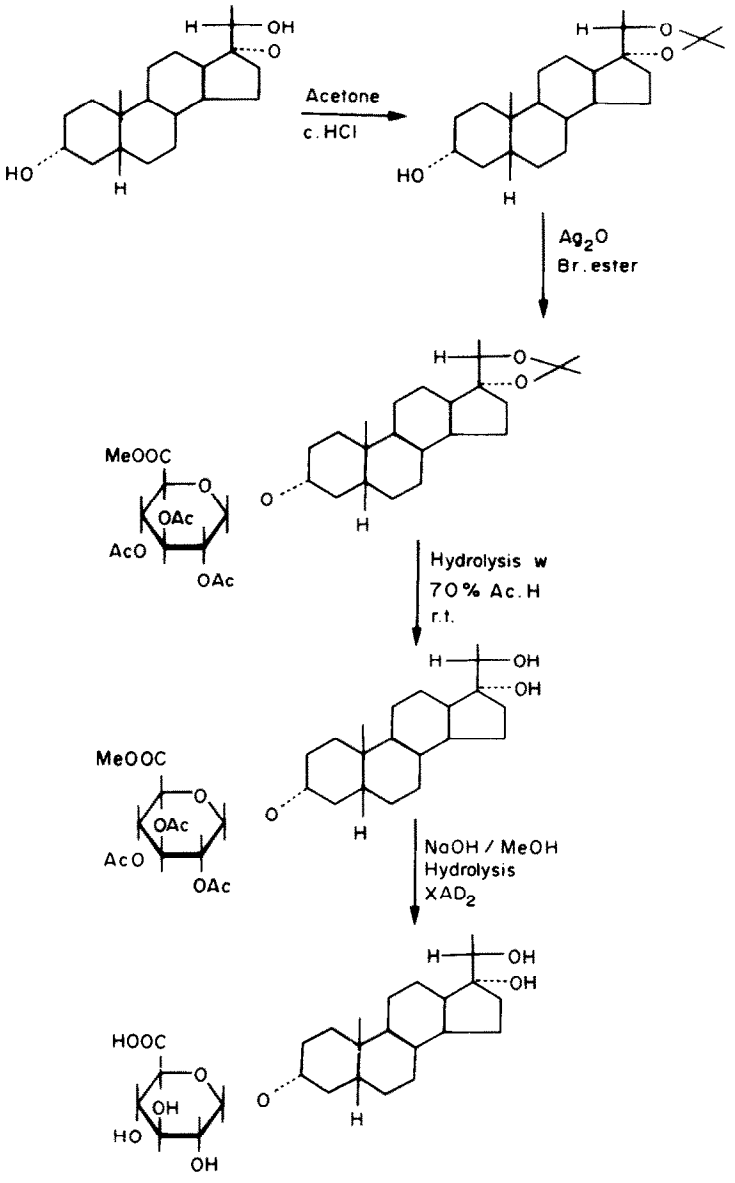

Fig. 1. Synthetic route for the preparation of 5β-pregnane-17,20α-diol-3α-yl-glucuronide.

plied by Kodak, Kirkby, Liverpool, L33 7UF, U.K. and activated charcoal (Norit A) was from Hopkin and Williams, Romford, Essex RM1 1HA, U.K. Bovine serum albumin (crystallized and freeze-dried) was obtained from Sigma (London) Chemical Co., Kingston, Surrey, KT2 7BH, U.K., and Freund's complete adjuvant was from Difco Laboratories, West Molesey, Surrey, KT8 0SE, U.K.

Celite 535 (Johns-Manville, London SE1, U.K.) for partition chromatography was extensively washed with concentrated HCl, water and methanol before being dried at 60°C. Thin-layer chromatography was carried out on prepared silica gel plates (Kieselgel 60 F_{254}; Merck) supplied by BDH. Sephadex G-25 was obtained from Pharmacia (G.B.) Ltd, Milton Keynes, MK9 3HP, U.K. Components of the liquid scintillant (2,5,diphenyloxazole (PPO), 1,4-di-2-(5-phenyloxazolyl)benzene (POPOP), Triton X-100 were purchased from Fisons Scientific Apparatus, Loughborough, Leics, LE11 0RG, U.K. Unless otherwise indicated chemical reagents were of analytical grade and were obtained from BDH. Tritium gas was supplied by the Amersham International PLC, Buckinghamshire, HP7 9LL, U.K.

Synthesis of 5β-pregnane-17,20α-diol-3α-yl glucuronide (hapten) [4]

Figure 1 illustrates the synthesis of pregnanetriol 3α-glucuronide starting from pregnanetriol (i.e. 5β-pregnane-3α,17,20α-triol). The starting material, 20α-triol was synthesized from 17-acetoxy-progesterone via a synthetic route established by Cooley *et*

al.[5]. After blocking two of the hydroxyl groups by forming a 17α,20α acetonide, in the presence of acetone and hydrochloric acid, the glucuronide radical was introduced at the C-3α position by the method of Schneider *et al.*,[6]. After the removal of the protective acetonide grouping with 70% (v/v) acetic acid at room temperature, the glucuronide triacetate methyl ester ("GAME") was hydrolyzed with methanolic sodium hydroxide to yield the desired glucuronide, 5β-pregnane-17,20α-diol-3α-yl glucuronide.

Synthesis of labelled 5β-pregnanetriol 3α glucuronide (radioligand) [6,7-^3H] 5β-pregnane-20α-ol-3α-yl glucuronide [4]

The synthesis of tritium-labelled pregnanetriol-3α-glucuronide ([6,7-^{3}H]5β-pregnane-17,20α-diol-3α-yl glucuronide) was carried out by the catalytic reduction of Δ^6-pregnene-triol 3α-glucuronide with carrier free tritium in the presence of palladium on charcoal (Fig. 2). The tritium-labelled glucuronide ([6,7-^{3}H]5β-pregnane-17,20α-diol-3α-yl glucuronide) was purified

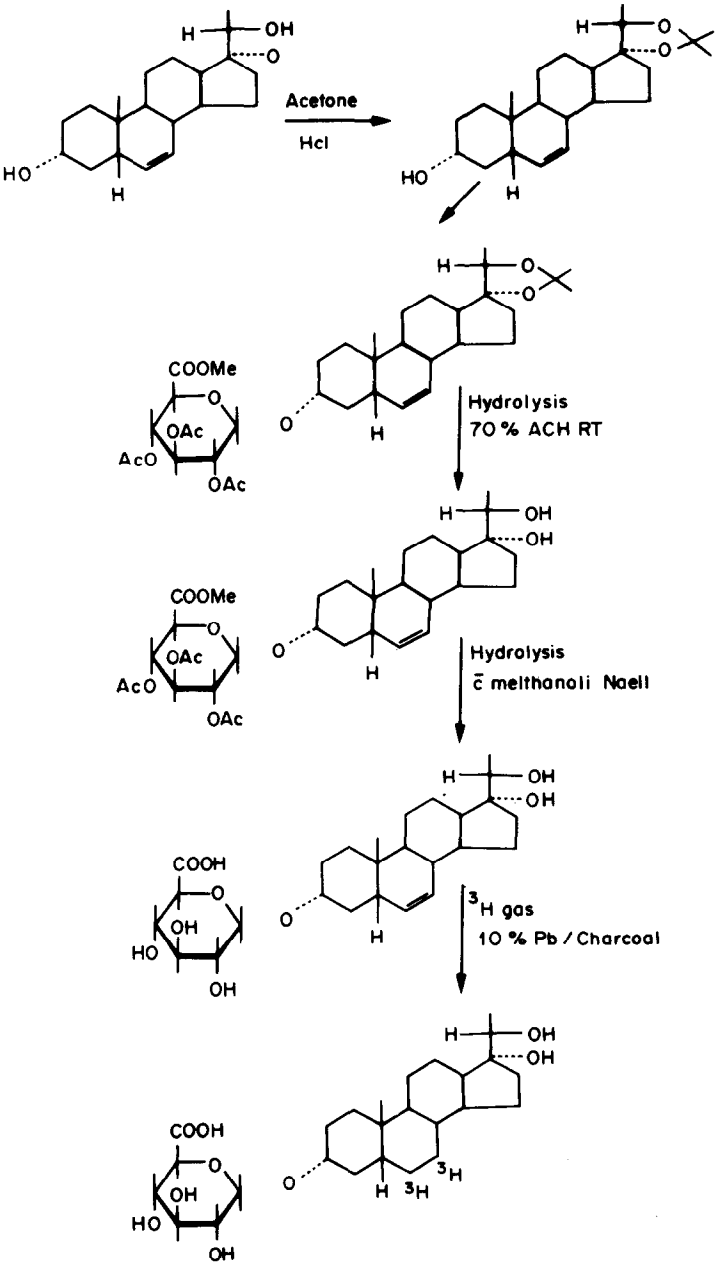

Fig. 2. Synthetic route for the preparation of [6,7-^{3}H]5β-pregnane-17,20α-diol-3α-yl-glucuronide.

by partition chromatography on Celite. The system employed for partition was 2 methyl-propan-2-ol; 1:2 dichloro-ethane; acetic acid and water (25:75:30:70, by vol). The specific radioactivity of the tritium labelled pregnanetriol 3α-glucuronide estimated to be 96% pure was 22 Ci/mmol.

The immunogen

The immunogenic complex of pregnanetriol 3α-glucuronide-BSA was prepared by the mixed acid anhydride reaction [7, 8]. The composition of the pregnanetriol 3α-glucuronide BSA complex was established by using [6,7-^{3}H]pregnanetriol 3α-glucuronide as a marker, and counting the radioactivity (i.e. [6,7-^{3}H]5β-pregnane-17,20α-diol-3α-yl glucuronide). The protein (BSA) content of the complex was determined by the method of Lowry *et al.*[9]. Analysis of the immunogen indicated a molar incorporation of the steroid glucuronide into the serum albumin of 16 mol/mol of protein corresponding to 27% of the theoretical maximum (60 amino residues).

RESULTS

The antiserum

Antisera to 5β-pregnane-17,20α-diol-3α-yl glucuronide–BSA immunogenic complex were raised in 3 adult male New Zealand white rabbits (body weight ~3 kg) as previously described [10]. The antibody titre of the serum samples obtained from the blood of the immunized rabbits were examined by constructing conventional antiserum dilution curves as described earlier [11]. The results were expressed graphically by constructing a serum dilution curve, in which the percentage binding of the radioligand is plotted against the log of the antiserum dilution. At a final antiserum dilution of 1/18,000, 1 pmol of the conjugate reduced the binding from 65% to approx. 20% thus providing the basis of a useful titre for the radioimmunoassay of 5β-pregnane-17,20α-diol-3α-yl-glucuronide.

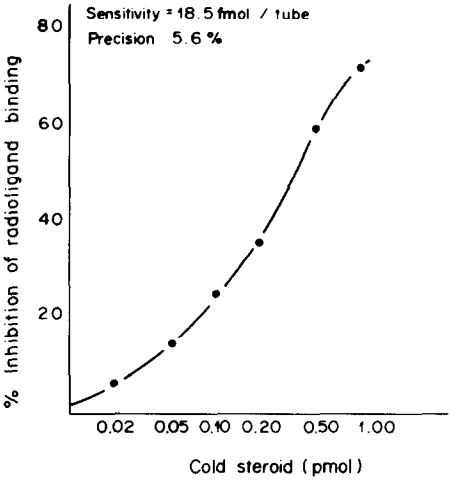

Fig. 3.

Calibration and specificity of the antiserum

Calibration and cross-reactivity tests for the radioimmunoassay of 5β-pregnane-17,20α-diol-3α-yl glucuronide were set up by incubating the diluted serum (1/18,000) in phosphate gelatine buffer with the radioligand [6,7-^{3}H]5β-pregnane-17,20α-diol-3α-yl-glucuronide (~10 nCi) and a range of non-radioactive ligand (0–1 pmol) at 37°C for 1 h. Unbound conjugate was removed by dextran coated charcoal suspension 200 μl; Norit A (0.25 g) dextran (0.025 g) in phosphate gelatine buffer (100 ml) and the bound radioactivity determined by β-scintillation counting of 250 μl aliquot [11]. The calibration graph (Fig. 3) was obtained by plotting the log of the amount of non-radioactive conjugate against the percentage inhibition of radioligand binding [12]. Table 1 illustrates the cross-reactivity of anti-5β-pregnane-17,20α-diol-3α-yl-glucuronide serum against other steroid conjugates and free steroids. The only compound which gave a measurable cross-reaction was the free steroid 5β-pregnane-3α,17,20α-triol ("pregnanetriol") which cross-reacted strongly (62.5%).

Table 1. Cross-reactions of anti-5β-pregnanetriol-3α-glucuronide serum against steroid glucuronides and free steroids

Compound	% Cross-reaction*
Steroid glucuronides	
1. 5β-Pregn-17,20α-diol-3α-yl-glucuronide	100
2. 5β-Pregn-20α-ol-3α-yl-glucuronide	Nil
3. 5β-Pregn-20-one-3α-yl-glucuronide	Nil
4. 5β-Pregn-20-one-3β-yl-glucuronide	Nil
5. 5α-Androstan-17-one-3α-yl-glucuronide	Nil
6. 3α-Androstan-17-one-3β-yl-glucuronide	Nil
7. 5β-Androstan-17-one-3α-yl-glucuronide	Nil
Free steroids	
1. 5β-Pregn-3α-17,20α-triol (Pregnanetriol)	62.5
2. 5β-Pregn-3α,20α-diol (Pregnanediol)	Nil
3. 3α-OH-5β-Pregn-20-one	Nil
4. 3β-OH-5β-Pregn-20-one	Nil

Nil = ⩽0.1%.

*Defined according to Thorneycroft *et al.*, (1970) as $(x/y) \times 100$ where x is the mass of the unlabelled steroid conjugate and y is the mass of the heterologous compound required to produce 50% inhibition of the binding of labelled conjugate by antibody.

Table 2. The concentration of pregnanetriol-3α-glucuronide in urine

Subject	Age	17-OH.P*	Testosterone nmol/l	0.I Index	Pregnane triol glucuronide μmol/l
AAA	4	387	4.4	1.7	150
BBB	6	137	1.6	1.6	120
CCC	10 months	375	—	2.0	180
DDD	7	375	—	—	180
EEE					132
FFF					170
GGG					80
HHH					80
JJJ	6 weeks				170

Normal: *(Plasma17-hydroxyprogesterone) 17-OH-P 30 nmol/l O.I Index Upper Limit 0.7.

Application of the RIA in clinical practice—Congenital Adrenal Hyperplasia (CAH)

Recognition of the clinical signs of congenital adrenal hyperplasia (CAH) presents little difficulty and the diagnosis can be confirmed readily by biochemical measurement e.g. plasma 17-hydroxyprogesterone concentration. It had been hoped to monitor the treatment of an affected subject during therapy but this has not yet been possible. We were, however, fortunate to receive stored urine samples from the Institute of Child Health, University of London, from subjects before the beginning of cortisol acetate therapy. The results of applying the RIA to these samples are given in Table 2 which also includes additional biochemical information supplied. Plasma 17-hydroxyprogesterone concentration in each case was substantially above normal levels (30 nmol/l) and corresponding to these values the urinary pregnanetriol 3α-glucuronide concentrations were excessive (80–180 μmol/l). The "11-Oxygenation Index" (0.1) [13, 14] referred to in this table represents the ratio of two urinary steroid measurements, 11-deoxy-17-oxosteroid/11-oxy-17-oxosteroid, this ratio being raised in CAH when there is an excessive excretion of pregnanetriol [15]. Unfortunately urines from these subjects after treatment were not available and the data listed in Table 3 relate to other, but similar subjects, after cortisol acetate therapy. The urinary excretion of pregnanetriol 3α-glucuronide from four of these subjects (6) is within the normal range, but this cannot be said to be true of the two male subjects (LLL 6 years; NNN 11 years). Although the "Oxygenation Index" appears to be within normal limits, the problem of balancing hormonal components in these two subjects has not been solved especially in the case of subject NNN who is approaching puberty.

Urines from normal menstrual cycles

In connection with other studies, serial urine samples were available which had been collected from women (19–35 years) throughout complete menstrual cycles. In order to compare the excretion of

Table 3. Subjects treated with cortisol acetate

Subject	Sex	Age	0.I. Index	pg Pregnanetriol glucuronide μmol/l
KKK	F	14	0.1	8.6
LLL	M	6	0.3	95
MMM	F	8	0.1	2.9
NNN	M	11	0.4	70
PPP	F	16	0.1	5.8
RRR	F	6	0.1	10

Table 4. Urinary 5β-pregnanetriol-3α-glucuronide excretion in normal women during the periovulatory period (μmol/l)

	Follicular phase				Luteal phase					
Day	−3	−2	−1	Plasma LH peak Day 0	+1	+2	+3	Day plasma LH peak	Mean ±SD follicular phase	Mean ±SD luteal phase
Subject A	3.8	4.2	4.8	9.87	9.4	8.96	8.2	Day 13	4.26 ± 0.5	9.10 ± 0.7
Subject B	4.01	4.24	5.8	14.16	12.86	10.16	9.30	Day 16	4.68 ± 0.97	11.62 ± 2.27
Subject C	3.2	4.2	4.92	16.98	14.09	12.7	12.51	Day 15	4.10 ± 0.86	14.07 ± 2.06
Subject D	3.8	4.2	5.8	10.3	9.2	8.0	6.4	Day 13	4.6 ± 1.05	8.47 ± 1.67

Mean Follicular phase 4.41 ± 0.27; Luteal phase 10.8 ± 2.56

5β-pregnanetriol 3α-glucuronide during the follicuiar phase of the menstrual cycle with that of the luteal phase, several urine samples corresponding to the peri-ovulatory period have been analysed for pregnanetriol 3α-glucuronide concentration by the RIA method (Table 4). These results show clearly that concentrations of the conjugate, 5β-pregnanetriol-3α-glucuronide, during the follicular phase are consistently lower than those on the day of maximum plasma LH concentration and that the mean excretion during the follicular phase (4.41 μmol/l) is less than the mean of the luteal phase (10.8 μmol/l). Since the mean ±SD excretion of 5β-pregnanetriol 3α-glucuronide on day 0, +1, +2, +3 is greater than twice mean ±SD on day −1, −2 and −3, it can be considered as a significant increase which will indicate the beginning of the luteal phase. The highest concentration of pregnanetriol 3α-glucuronide encountered, 16.98 μmol/l, was very much lower than the values found in the urines of juveniles with untreated congenital adrenal hyperplasia (80–180 μmol/l).

DISCUSSION

Techniques similar to those described for steroid glucuronides [10, 11] led to the successful development of a RIA procedure for the measurement of 5β-pregnane-17,20α-diol-3α-yl glucuronide (pregnanetriol 3α-glucuronide). The antisera were induced using a synthetic hapten linked covalently to BSA by the mixed acid anhydride reaction [16]. Incorporation of 5β-pregnanetriol 3α-glucuronide into the carrier protein was 16 mol/mol of BSA and the resulting steroid conjugate–protein complex induced an antiserum with an antibody titre of 18,000. Under the conditions of the assay at a final dilution of 1/18,000 the non-radioactive conjugate (500 pg; lpmol) changed the binding from 67 to 25%.

The specificity of the antiserum against other steroid glucuronides tested was absolute, but it is noteworthy that there was a strong cross-reaction with free 5β-pregnane-3α,17,20α-triol (67%). It is widely believed that it is the portion of the hapten remote from the point of attachment to the protein carrier which determines the specificity of antibodies formed [17, 18] and the side chain structure of 5β-pregnane-17,20α-diol-3α-yl-glucuronide may perform this function.

The method has distinct advantages over the existing methods [13, 14, 19] used to diagnose and monitor therapy of CAH subjects. Considering the simplicity of the assay procedure it is concluded there there could be useful application in a variety of clinical conditions. Pregnanetriol appears to be formed to some extent in the ovary as well as in the adrenal cortex and a cyclic variation in excretion during the menstrual cycle has been observed [20, 21]. Burger and Sommerville [22], and independently Crooke *et al.*[23], showed that pregnanetriol formation was increased after gonadotropin stimulation. Since there is a very close temporal relationship between circulating levels of 17-OH progesterone (17-OH-P) and the mid cycle surge of luteinizing hormone (LH) [24–27] in healthy women with regular menstrual cycle, measurement of pregnanetriol 3α-glucuronide which is the chief metabolite of 17-OH-P will be a suitable marker for the detection of ovulation.

A multicentre study was undertaken by the World Health Organization to assess the urine excretion profile of pregnanetriol 3α-glucuronide in early morning (EMU) and 24 h urine sample in normal ovulating women to detect ovulation. (WHO report, Bedolla-Tovar *et al.*,[28] in press). In applying the method to normal menstrual cycle (4–10 μmol/l) substantial dilution of the samples was necessary before assay; with normal menstrual cycle urine dilution was 1000–2000-fold with correspondingly high dilution for abnormal samples. Makin *et al.*,[29] found a progressive increase in "11-Oxygenation Index" (O.I) throughout pregnancy and a major contribution to this increase was attributed to pregnanetriol [30] and independently Harkness and Love[31] showed that there was a sharp decrease in the excretion of 17-hydroxycorticosteroids on the day of delivery. These observations suggest that the measurement of urinary pregnanetriol 3α-glucuronide might be used to monitor the well-being of the foeto–placental unit throughout pregnancy.

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