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SOLID-PHASE RADIOIMMUNOASSAYS OF ESTRIOL-16α-GLUCURONIDE IN URINE AND PREGNANCY PLASMA

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SUMMARY

Simple and specific solid-phase radioimmunoassays are described for the determination of estriol-16α-glucuronide in urine and pregnancy plasma. The radioimmunoassay is carried out directly in diluted urine or plasma. The antiserum-bound steroid is separated from the unbound fraction simply by centrifugation, and in the method used for pregnancy urine the supernatant is used for radioactivity counting. In the modification for plasma and non-pregnancy urine the antiserum-bound steroid is freed from the solid support by shaking with hydrochloric acid, and the freed radioactivity is counted. Antiestriol-16α-glucuronide antiserum was raised in a sheep and coupled to agarose gel. The antiserum showed exceptionally good specificity. Data on the reliability of the methods are presented and reference values for urinary estriol-16α-glucuronide in pregnancy urine are given. The methods seem suitable for monitoring of foetal well-being in pregnancy and for prediction of ovulation in fertile women.

INTRODUCTION

Conventional techniques for measuring conjugated steroids in urine or plasma involve hydrolysis and extraction of the free steroid. Recently, radioimmunological methods for measuring the intact conjugates have been developed [1–5]. In one study the azobenzoyl derivative of estriol-16α-glucuronide coupled mainly to C-4 was used for immunization [6] but the exact procedure has not been published. Most antibodies were raised with antigens in which the steroid glucuronide was coupled to the macromolecule through the carboxyl group of the glucuronic acid moiety. In antibody production coupling to other positions of the steroid would theoretically yield more specific antibodies; however, direct coupling is much simpler and more convenient, and in our hands has resulted in highly specific antibodies. Utilizing antibodies to estriol-16α-glucuronide (E_3-16αG), we have developed solid-phase radioimmunological methods for determination of this steroid in urine and pregnancy plasma. The methods are suggested to be used for prediction of ovulation in non-pregnant women and for the monitoring of foetal well-being.

EXPERIMENTAL

Material. Twenty-four h urine was collected from normal pregnant women from the eighth week of gestation to term. In some cases plasma samples were taken into heparinized tubes the morning when urine collection was finished. Urine was also collected from volunteers during a menstrual cycle for determination of E_3-16αG concentration before and around ovulation. Ovulation was confirmed by assays of urinary LH and plasma progesterone. All urine and plasma samples were stored at −20°C until analysed.

Reference compounds. [6,9-^{3}H]-Estriol-16α-glucuronide was purchased from the Radiochemical Centre (Amersham, Bucks, England), S.A. 32 Ci/mmol. The following reference steroids were used: estriol-16α-glucuronide (E_3-16αG), estriol (E_3), estradiol-17β (E_2), estrone (E_1) and 16-oxo-estradiol-17β (16-oxoE_2) were from Ikapharm (Ramat-Gan, Israel). Estrone-3-glucuronide, (E_1-3G), estradiol-3-glucuronide (E_2-3G), estriol-3-glucuronide (E_3-3G), estradiol-17β-glucuronide (E_2-17βG), 17-epiestriol-16α-glucuronide (17-epiE_3-16αG), and 16-epiestriol-16β-glucuronide (16-epiE_3-16βG) were gifts from Dr. A. E. Kellie. 2-hydroxyestradiol-1-S-glutathione (2-OH-E_2-1-S-Glut.) was a gift from Dr. J. C. Elce. Estrone-3-sulphate (E_1-3-S) was purchased from Orion Oy (Helsinki, Finland) and estriol-3-sulphate (E_3-3-S) from Leo Ab (Hälsingborg, Sweden).

Reagents. Tricine (N-Tris-(hydroxymethyl)methylglycine)(Fluka AG, Bucks, Switzerland), sodium chloride, sodium hydroxide, sodium azide and hydrochloric acid (Merck AG, Darmstadt, Germany), gelatine (Difco Laboratories, Detroit, Michigan, U.S.A.), 2,5-diphenyloxazole (PPO)(Koch Light Laboratories, Colnbrook, Bucks, England), 1,4-bis-2(5-phenyloxazolyl)benzene (POPOP) (Packar Instrument Company Inc., Downers Grove, U.S.A.), Triton-X-100 (BDH Chemicals Ltd., Poole, England), and xylene (J. T. Baker Chemical Co., Deventer, Holland).

Assay buffer. Tricine-buffered saline (TBS), pH 8.0, containing 0.1% gelatine was prepared as described by Hennam *et al.* [7].

Antiserum. E_3-16αG was coupled to thyroglobulin *via* the carboxyl group of glucuronic acid as described

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by Abraham and Grover [8] and the macromolecule conjugate was injected into two sheep. The IgG fraction was isolated by precipitation with sodium sulphate and immobilized by reaction with CNBr-activated Sepharose® (Pharmacia Fine Chemicals Ab, Uppsala, Sweden): 1 g of Sepharose 4B is swelled and washed for 15 min on a glass filter with 10^{-3} M HCl solution (200 ml). 1 ml of IgG solution, diluted to 5 ml with 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl, is mixed with the gel in the test tube and the mixture is rotated for 2 h at room temperature or overnight at 4°C. Unbound material is washed away with coupling buffer and any remaining active groups are reacted with 1 M ethanolamine at pH 8 for 1–2 h. Three washing cycles are used to remove non-covalently absorbed protein, each cycle consisting of a wash at pH 4 (0.1 M acetate buffer containing 1 M NaCl) followed by a wash at pH 8 (0.1 M borate buffer containing 1 M NaCl). The gel is stored at +4°C.

Before use in radioimmunoassay a suitable amount of the gel is washed with 0.9% sodium chloride to remove any free antiserum displaced from the gel. The gel suspension is diluted to 1:500 (antiserum A) or 1:2000 (antiserum B) for the assay. These dilutions correspond to 1:12,500 and 1:5000 dilutions of original antisera.

Scintillation fluid. PPO-xylene was prepared by dissolving 15 g of PPO and 1 g of POPOP in 1250 ml of Triton X-100 and xylene added 5000 ml.

Methods

Method for pregnancy urine (method I). 24-h urine samples are diluted 1000–10,000 times with the assay buffer and 0.5 ml aliquots are transferred in duplicate to disposable Eppendorf microtubes. A standard curve is constructed by diluting E_3-16αG to 25–2000 pg/ml with the assay buffer and assayed as the samples. 0.1 ml of [^{3}H]E_3-16αG dilution containing about 100 pg (7.1 nCi) is added to the tubes. An aliquot of the antiserum suspension is washed twice in a centrifuge tube by adding 10 ml of saline and then centrifuging (10 min, 4000 *g*). The washed suspension is diluted to produce about 40–50% binding of the tracer in the absence of unlabelled ligand. The antiserum suspension is continuously mixed with a magnetic stirrer and 0.4 ml added to the tubes with an automatic pipette, and the reaction mixture is incubated for 2 h at room temperature with gentle rotation. The tubes are centrifuged at room temperature (2.5 min, 8000 *g*), and 0.7 ml aliquot of the supernatant from each tube is transferred to the counting vial containing PPO-xylene, and the radioactivity in each tube is measured in a Wallac 81000 automatic liquid scintillation counter (Wallac Oy, Turku, Finland).

The incubation time of 2 h was chosen after testing 30 min, 1, 2, 3 and 24 h incubation times. Standard curves (5 tubes for each point of the standard curve) were compared. The sensitivity and precision of each curve was calculated and found that the 2-h incubation did not differ from 3- or 24-h incubation. 30 min and 1 h did not yield satisfactory standard curves.

A modification for pregnancy plasma and non-pregnancy urine (method II). Plasma is diluted 50–200 times and non-pregnancy urine 10–50 times with the assay buffer and assayed in the same way as pregnancy urine (see above) to the point after separation of the antiserum-bound and unbound fractions. After centrifugation the bulk of the supernatant is removed by suction and the particles (0.2 ml) containing the antiserum-bound steroid are washed twice with 1 ml of 0.9% sodium chloride. 0.8 ml of 1 mol/l. HCl is added and the tubes are shaken for 30 min in an Eppendorf rotamixer 3300 to liberate the radioactivity from the particles. After centrifugation 0.7 ml of the supernatant is taken for radioactivity counting. It may be mentioned that no dissociation of antibody–ligand complex caused by washing with NaCl was ever found.

Estriol-16α-glucuronide [9] and *estriol* [10] were also assayed in pregnancy urine by specific gas-chromatography methods. Progesterone was assayed by radioimmunoassay from 0.5 ml of plasma which was extracted twice with five vol. of petroleum ether. In other respects the previously published method was followed [11].

LH in urine was assayed by a radioimmunological method. The extraction method of Reiter *et al.* [12] was slightly modified with smaller volumes. The LH is precipitated with 1 ml of cold acetone from 5 ml of acidified urine (pH 4.5 is obtained with glacial acetic acid). The urine is thoroughly mixed with a Vortex shaker and kept overnight at +4°C. The supernatant was poured off and the acetone still left in the precipitate is evaporated. The immunoassay is carried out with a double antibody solid-phase technique (DASP)(Karonen *et al.*, manuscript in preparation). The 2nd IRP HMG standard (C 67/161) was used as standard. (WHO International Laboratory for Biological Standards, National Institute for Medical Research, Mill Hill, London, N.W.7, England).

RESULTS

Specificity. Known amounts of estriol-16α-glucuronide, estriol (E_3), estradiol (E_2), estrone (E_1), E_3-3G, E_3-3-S, 16-epiE_3-16βG, 17-epiE_3-16αG, E_1-3-S, E_1-3G, E_2-17βG, E_2-3G, 16-oxoE_2 and 2-OH-E_2-1-S-glut. were dissolved in the assay buffer and suitably diluted. Cross-reactivity of each steroid were determined at 50% inhibition of the binding of labelled E_3-16αG [13]. The antiserum (A) used in the assays did not cross-react significantly with any of these steroids: less than 1% with the conjugates and 0.2–2.0% with the free steroids. Table 1 shows the percentage cross-reactivities of the two Sepharose-coupled antisera (A and B) with the steroids listed above. Also presented for comparison the specificity of the antiserum (C) used previously in the liquid-phase RIA of E_3-16αG [4].

Table 1. Cross-reactivities of the two antisera, A and B, used in the solid-phase RIA for urine and plasma and of antiserum C used in the liquid-phase RIA [4]

Steroid	A	B	C
Estriol-16α-glucuronide	100	100	100
Estriol	2.0	1.3	<1
Estradiol	1.5	0.4	<1
Estrone	0.2	<0.1	<1
Estriol-3-glucuronide	<0.1	<0.1	<0.1
Estriol-3-sulphate	<0.1	<0.1	3.0
16-Epiestriol-16β-glucuronide	<0.1	0.6	0
17-Epiestriol-16α-glucuronide	0.6	17.2	0.1
Estrone-3-glucuronide	<0.1	0.1	0
Estrone-3-sulphate	<0.1	<0.1	<1
Estradiol-17β-glucuronide	0.1	0.9	<1
Estradiol-3-glucuronide	0.2	<0.1	<1
16-Oxoestradiol-17β	0.9	<0.1	—
2-Hydroxyestradiol-1-S-glutathione	0.4	0.1	<1

The affinity constant of the antiserum (A) was determined from a Scatchard plot [14] and by a saturation curve method suggested by Odell *et al.* [15]. The two methods gave results of the same order: 1.12×10^{10} l/mol by the former and 1.15×10^{10} l/mol by the latter method.

Accuracy was determined by adding 50–500 pg of E_3-16αG to a pooled diluted late-pregnancy urine sample. The samples were assayed, and the recoveries for each addition were calculated. The mean recovery was $95.7 \pm 9.7\%$ ($N = 20$) by method I and $101.7 \pm 9.3\%$ ($N = 20$) by method II. There were no significant differences of the mean recoveries for each level of addition.

The pooled urine sample was analyzed by both methods 10 times at different occasions. The mean value obtained by method II was 1.9% smaller than by method I and the difference was not significant.

Precision. Intra- and interassay coefficient of variation (C.V.) were calculated according to Snedecor[16] for both methods from 15 urine samples diluted to 100–500 pg/0.5 ml. The interassay C.V. was 8.3% for method I when calculated for these 15 samples in two different assays. When these samples were pooled and assayed in 10 different assays, the interassay C.V. was 8.1% for method I and 8.0% for method II. The intra-assay coefficient of variation was 7.3% for method I and 6.9% for method II.

Sensitivity. By both methods the lowest detectable value which differs from zero by two standard deviations was 25 pg. A graphical presentation of logit–log transformation of a typical standard curve is shown in Fig. 1.

The day-to-day variation in urinary excretion of E_3-16αG in pregnancy was calculated from 26 pairs of 24-h urine samples collected on consecutive days. Variation was 12.4%, which is in good agreement with the figure of 10.2% previously obtained [4].

Reference values for E_3-16αG concentration in pregnancy urine were determined in 100 urine samples from the 8th week of gestation to term. The 24-h values calculated as mg estriol are presented in Fig. 2.

Comparison of the results with estriol-16α-glucuronide and estriol values determined by gas chromatographic (GLC) methods

E_3-16αG was determined in 25 urine samples collected from normal pregnant women. Urines were assayed by the present method (I) and by a GLC method [9]. A good correlation was found, the coefficient of correlation being 0.97. The regression equation was $y = 0.92\,x + 0.89$, where y represents the values obtained by RIA and x those by GLC. The mean values for E_3-16αG excretion (as E_3) were 20.4 mg/24 h by RIA and 21.2 mg/24 h by GLC. The correlation of these methods is shown in Fig. 3. Table 2 allows a comparison of E_3-16αG values obtained for one pregnant woman by the GLC method [9], by the present solid phase RIA and by the RIA previously described [4].

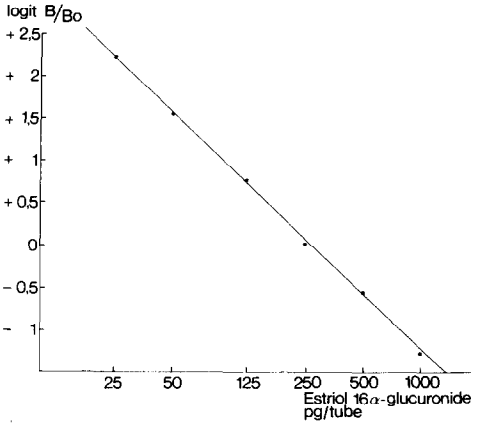

Fig. 1. Logit–log transformation of a dose response curve of E_3-16α-glucuronide (pg/tube) logit $B/B_0 = B/B_0/(1 - B/B_0)$. B = the bound, labelled ligand, B_0 = the bound, labelled ligand at zero dose of unlabelled ligand, B/B_0 = the percentage bound.

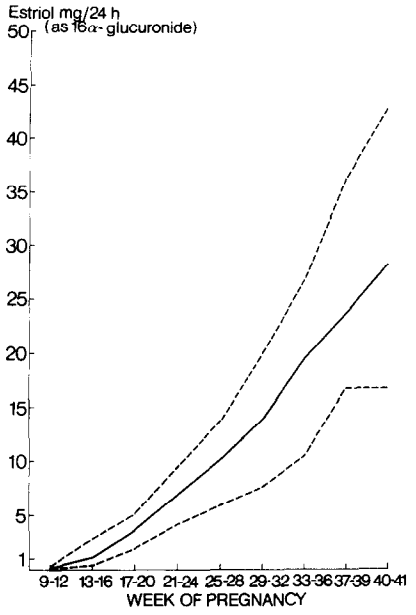

Fig. 2. E_3-16α-glucuronide concentration (expressed as estriol mg/24 h) in 100 normal pregnancy urine samples from the 8th week of gestation to term. All values fall between the dotted lines.

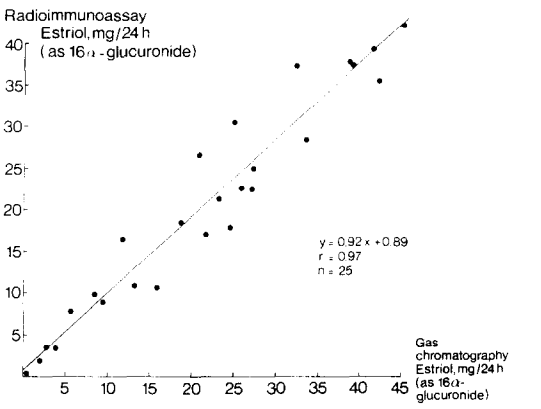

Fig. 3. The correlation between a specific gas chromatographic method for estriol-16α-glucuronide [9] and the present solid-phase radioimmunoassay for estriol-16α-glucuronide.

E_3-16αG values of 22 third-trimester 24-h urine samples were also compared with the total estriol values of these samples determined by a gas chromatographic method after hydrolysis [10]. The coefficient of correlation was 0.95 and the regression equation $y = 0.92\,x + 0.40$, where y represents the values obtained by RIA and x those by GLC. $\bar{x} = 23.6$ mg/24 h and $\bar{y} = 22.2$ mg/24 h (Fig. 4).

Preliminary results of urinary E_3-16αG excretion during the menstrual cycle

Assays of E_3-16αG were made by method II on 24-h urine samples from three normally menstruating women. In subject No. 1 (Fig. 5) the peak value of E_2-16αG was reached one day before ovulation took place, as judged by assays of urinary LH. In this subject the rise in E_3-16αG concentration started 5 days before ovulation. In subject No. 2 the peak value of E_3-16αG was reached on the same day as ovulation took place, but the rise had started 7 days before. In subject No. 3 the peak value was obtained one day before ovulation, the rise having started 4 days earlier. It can also be observed that the amount of

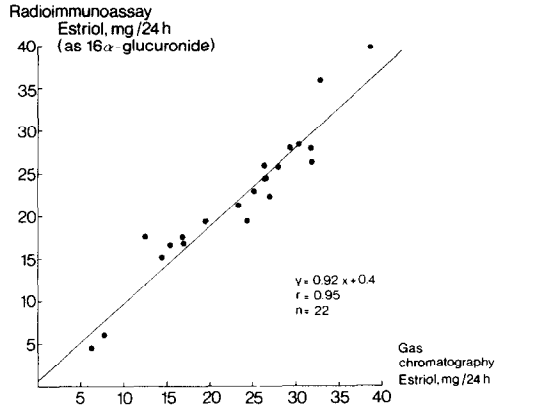

Fig. 4. The correlation between a gas chromatographic method for estriol [10] and the present solid-phase radioimmunoassay for estriol-16α-glucuronide.

Table 2. Comparison of results (E_3 mg/24 h) obtained by assay of estriol-16α-glucuronide by three different methods in 11 urine samples from one woman covering her whole pregnancy

Sample No.	GLC reference method [13]	RIA (solid phase) antiserum A	RIA (liquid phase) antiserum C
1	0.5	0.5	0.8
2	3.8	3.7	4.0
3	9.3	9.0	14.0
4	13.4	10.9	21.4
5	18.8	18.5	19.7
6	21.0	26.8	39.0
7	27.5	25.2	31.1
8	25.3	30.6	32.2
9	39.7	37.8	41.8
10	45.7	42.7	62.7
11	39.1	38.1	54.5
Mean values	22.2	22.2	29.2

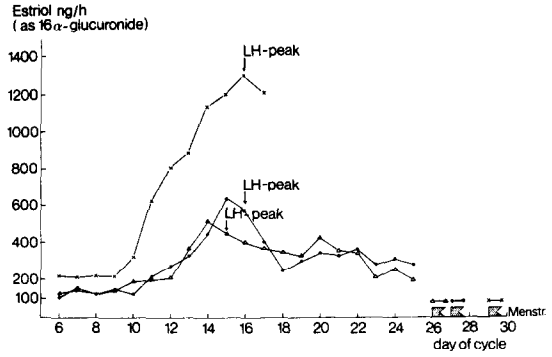

Fig. 5. Excretion of estriol-16α-glucuronide (expressed as estriol ng/h) in 24-h urine samples in three menstrual cycles. ●——● = subject No. 1, Δ——Δ = subject No. 2, ×——× = subject No. 3.

E_3-16αG excreted in the early follicular phase is remarkably constant.

Preliminary results of plasma E_3-16αG assays in pregnancy

In Table 3 plasma E_3-16αG values obtained in two normal pregnant women are shown.

DISCUSSION

The purpose of this study was to develop a rapid and reliable method for prediction of ovulation in non-pregnant women as well as for monitoring foetal well-being in pregnancy. The advantage of measuring estriol-16α-glucuronide instead of total estriol as an index of foetal well-being has been discussed earlier [4]. Although plasma samples are easier to obtain than 24-h urine samples and a satisfactory correlation has been found between urinary estrogens and plasma estriol [17, 18], assay of the latter hormone is not recommended as the sole method of monitoring estrogen metabolism in high-risk pregnancies [19]. Present methods of measuring estriol or total estrogens in urine usually involve hydrolysis and purification, which render them time-consuming and impractical and result in procedural losses. Direct measurement of estrogen conjugates would be preferable, and some methods have already been published. Of the existing immunoassays which measure estriol-16α-glucuronide in pregnancy urine, one is based on charcoal-dextran separation [4] and another on ammonium sulphate precipitation [3]. A solid-phase immunoassay previously published utilized polyacrylamide as a solid support for antiestriol-16α-glucuronide antiserum [2]. These radio-ligand assays of estriol-16α-glucuronide, though suitable for clinical purposes, were not completely specific for E_3-16αG [2, 3] or, despite high specificity of the antiserum confirmed by testing the percentage cross-reactivity with related compounds, the values given by the radioimmunoassay were too high as compared with those obtained with an accurate and specific GLC procedure [4]. The antiserum used in the present method showed good specificity in cross-reactivity studies, and in addition direct proof of specificity was obtained, because the results correlated well with those of the same above-mentioned specific and accurate method based on gas-chromatography [9]. This GLC method measures all four estriol conjugates separately and the results suggest that the new procedures for E_3-16αG assay does not include measurement of the double conjugate E_3-3-sulphate-16αG. However, this conjugate was not available for cross-reactivity studies. E_3-3-sulphate-16αG showed a 36% cross-reactivity in the method of Soares *et al.*[3]. The values obtained for urinary estriol-16α-glucuronide in late pregnancy were only slightly lower than the total estriol values given by another GLC method [10], although at that stage the conjugate has been reported to comprise 70–80% of total estriol [20, 21]. This is because in this GLC method [10] no correc-

Table 3. Determinations of estriol-16α-glucuronide (expressed as estriol ng/ml) in plasma samples from two normal pregnant women

Weeks of pregnancy	E_3 ng/ml (E_3-16α-glucuronide) Subject No. 1	Subject No. 2	Weeks of pregnancy	E_3 ng/ml (E_3-16α-glucuronide) Subject No. 1	Subject No. 2
8	0.47		25		
9	0.53		26	14.5	
10			27	12.4	27.3
11	0.68		28		33.9
12			29		39.0
13	1.22	1.01	30	15.5	30.8
14		2.18	31		31.5
15	1.92	3.97	32	16.4	31.2
16		3.70	33	17.1	
17	4.34	6.57	34	19.1	51.8
18		6.75	35	20.7	52.4
19	3.51	8.52	36		66.4
20		6.04	37	30.6	55.9
21	4.93	17.1	38	37.1	73.3
22			39		80.4
23			40		70.8
24			24 h after delivery		23.5

tion is made for the chemical losses during hydrolysis and purification in the assay of estriol. Thus in our view the present method does not measure any other compounds except estriol-16α-glucuronide.

One of the disadvantages of measuring total estriol in pregnancy urine is the considerable daily fluctuation (18–50%) [22–26]. The day-to-day variation of estriol-16α-glucuronide excretion seems to be somewhat less, 12.4%. This is partly due to the fact that other constituents of urine and drugs do not interfere with the determination. In addition, the clearance of the conjugate is very high, approximating that of para-aminohippuric acid [21]. Thus its urinary excretion should not be greatly influenced by alteration in kidney function.

The present solid-phase RIA in pregnancy urine is quick and simple, and can be performed in one working day. Since the antiserum is coupled to a solid support, separation of the antiserum-bound fractions is rapid and needs no refrigeration. The method for plasma and non-pregnancy urine, though a little more time-consuming, can be performed in 1 working day. Because the method involves no inherent procedural losses, no radioactive internal standard is necessary.

In the normal non-pregnancy urine and in pregnancy plasma the concentration of estriol-16α-glucuronide is too low for direct radioimmunoassay with the present antibody in solution, because of the interference of other constituents in the fluids with the method. Different dilutions of the fluids gave different results. Thus in work with the low dilutions present in non-pregnancy urine and pregnancy plasma washing of the particles which contain the antiserum-bound steroid and release of the radioactivity with acid seem necessary. The present method failed to measure the conjugate accurately in non-pregnant urine and plasma when the supernatant (the unbound fraction) was used for liquid scintillation counting. By this direct solid-phase radioimmunoassay estriol-16α-glucuronide could be measured in plasma at a dilution of 1:5 as early as in the eighth week of gestation. The conjugate could also be measured without difficulty through the menstrual cycle even in the beginning of the follicular phase when the level of estrogen is low. The excretion of estriol-16α-glucuronide seems to be fairly constant at the beginning of the cycle until the rise before ovulation starts. A distinct rise of the concentration from the basal level was seen 4–7 days before ovulation took place. Thus it may be possible to use this rapid method for the prediction of ovulation in women.

The preliminary results of estriol-16α-glucuronide measurement in monitoring of foetal well-being have been published [27].

Although the methods presented in this paper are both fairly rapid, the slowest step is still liquid scintillation counting. To speed up the counting time we are trying to couple iodine to estriol-16α-glucuronide and use it as a tracer instead of the tritiated conjugate.

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