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RADIOIMMUNOASSAY OF ESTRIOL-16-GLUCURONIDE USING TRITIATED AND RADIOIODINATED RADIOLIGANDS: DIRECT RADIOIMMUNOASSAY OF URINARY ESTRIOL-16-GLUCURONIDE DURING THE MENSTRUAL CYCLE

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SUMMARY

Estriol-16-glucuronide-[^{125}I]-iodohistamine (E_3-16G-[^{125}I]) was prepared and utilized in a radioimmunoassay (RIA) in conjunction with anti-estriol-16-glucuronide-bovine serum albumin (E_3-16G-BSA) serum (RIA No. 1). This RIA was then compared with two other RIA methods employing [^{3}H]-labeled estriol-16-glucuronide (E_3-16G) together with either anti-E_3-16G-BSA serum (RIA No. 2) or anti-estriol-16-glucuronide-2-azobenzoic acid-bovine serum albumin (E_3-16G-2-ABA-BSA) serum (RIA No. 3). All three RIA's were accurate and precise, however, they differed in assay sensitivity and specificity. Most sensitive was RIA No. 1 and least sensitive was RIA No. 3. Both RIA's No. 1 and 2 were less specific than RIA No. 3 since unconjugated estriol and estrone-3-sulfate exhibited large and comparable cross-reactions in RIA's No. 1 and 2, averaging 50% and 41%, respectively. Furthermore, measurement of E_3-16G in 10 different urine aliquots collected from non-pregnant women employing RIA's No. 1–3 showed that RIA's No. 1 and 2 yielded comparable results, however, the results obtained by RIA No. 3 were, on the average, 26% lower than the mean of the values measured with RIA's No. 1 and 2. Consequently, RIA No. 3 was used to measure daily 24-hour urinary E_3-16G excretion in 7 women throughout an entire menstrual cycle. These data are in agreement with colorimetric and fluorimetric estimates of 24-h urinary estriol values throughout the menstrual cycle. The preovulatory rise of urinary E_3-16G excretion, as quantitated by this RIA, is comparable to that of serum estradiol measured in the same 7 women, but peaks 1 to 2 days later than the serum estradiol surge.

INTRODUCTION

Several radioimmunoassay (RIA) methods for the measurement of urinary estriol-16-glucuronide (E_3-16G) have recently been reported [1–7]. In each of these procedures, the authors employed antisera against E_3-16G protein conjugates in combination with a [^{3}H]-labeled radioligand. Although the use of tritiated radioligands permits the establishment of specific and sensitive RIA methods, utilizing [^{3}H]-labeled radioligands requires longer counting times as well as expensive scintillation fluid and counting vials in comparison with radioiodinated radioligands. Moreover, γ-counters are more readily available than liquid scintillation counters. Therefore, the present study was undertaken to develop and test a RIA procedure for urinary E_3-16G determinations which would employ [^{125}I]-labeled E_3-16G as radioligand. Estriol-16-glucuronide-[^{125}I]-iodohistamine (E_3-16G-[^{125}I]) was evaluated in conjunction with two different antisera for its properties as a radioligand for the RIA of E_3-16G. A RIA utilizing E_3-16G-[^{125}I] and an anti-estriol-16-glucuronide-bovine serum albumin (E_3-16G-BSA) serum was established and compared with two other RIA methods employing [^{3}H]-labeled E_3-16G together with either anti-estriol-16-glucuronide-2-azobenzoic acid-bovine serum albumin (E_3-16G-2-ABA-BSA) serum or anti-E_3-16G-BSA serum. These three RIA's were compared and the most specific method was used to measure urinary E_3-16G excretion during the mentrual cycle by direct RIA.

EXPERIMENTAL

Subjects

Antecubital venous blood was obtained daily between 8 and 9 a.m. from healthy women during an entire menstrual cycle. The blood was allowed to clot. The serum was obtained by centrifugation and stored at −15°C. Twenty-four hour urine collections were obtained from all women throughout the same menstrual cycle during which daily blood samples were drawn. The urine was collected in plastic bottles containing 10 ml of a 10% (w/v) aqueous sodium azide solution and stored under refrigeration during the 24-h collection period. Upon completion of urine collection, urine aliquots were transferred into bottles

of borosilicate glass and stored at −15°C until they were analyzed by RIA.

Radioimmunoassay of serum estradiol and LH

Serum estradiol (E_2) was determined as previously described [8] using celite partition chromatography to separate the E_2 fraction from other estrogens and an anti-estradiol-17β-hemisuccinyl-bovine serum albumin serum for RIA. Serum LH was measured by a double-antibody RIA [9].

Radioimmunoassay of estriol-16-glucuronide

Steroids. Authentic E_3-16G was a gift of Dr. Willard Allen, St. Louis, MO. Estrone glucuronide, estradiol-3-glucuronide, estradiol-17β-glucuronide and estriol-3-glucuronide were gifts of Dr. Alexander E. Kellie, London, England. Estrone sulfate and estradiol-3-sulfate were obtained from AB Leo, Hälsingborg, Sweden, and estriol was obtained from Steraloids, Inc., Pawling, NY. [6,9-^{3}H]-E_3-16G with a specific activity of 30 Ci/mmol was purchased from Amersham Corporation, Arlington Heights, Illinois. E_3-16G-[^{125}I] was prepared according to the procedure described by Nars and Hunter[10] for estradiol-6-(O-carboxymethyl) oximino-[^{125}I]-iodohistamine and by us for progesterone-3-(O-carboxymethyl)oximino-[^{125}I]-iodohistamine with the exception that ethyl acetate was used to extract E_3-16G-[^{125}I] from the reaction mixture [11]. The E_3-16G-[^{125}I] was then purified by thin-layer chromatography (t.l.c.) on F-254 aluminum-backed silica gel plates (EM Laboratories, Inc., Elmsford, NY) in benzene–ethanol–acetic acid (75:24:1, by vol.). The peak of radioactive material with an R_F of 0.16 was eluted with methanol. The eluate was centrifuged to remove silica gel particles and stored at 4°C.

Antisera. Two different antisera were used in this study. Anti-estriol-16-glucuronide-2-azobenzoic acid-bovine serum albumin serum was obtained from Dr. Stanley Gross, Los Angeles, CA [2]. Anti-estriol-16-glucuronide-bovine serum albumin serum was a gift of Dr. Alexander E. Kellie, London, England.

Radioimmunoassays. Urinary E_3-16G was measured directly by RIA: 1.5 ml urine aliquots were centrifuged for 10 min at 3000 rev/min. to remove insoluble particles whereupon 0.5 ml was diluted with 4.5 ml of phosphate-gelatin buffer (PGB) consisting of 0.15 M sodium chloride and 0.1 M sodium phosphate with a pH of 7.0 and containing 0.1% (w/v) of both Knox unflavored gelatin and sodium azide. A 0.2 ml aliquot of the PGB-diluted urine was then transferred into a 10 × 75 mm RIA tube in duplicate. Authentic E_3-16G was dissolved in PGB to yield a stock solution of 20 ng/ml. This was serially diluted for standard curves which were included in each assay by transferring 0.2 ml aliquots from a sequence of concentrations of E_3-16G in PGB into 10 × 75 mm RIA tubes in duplicate.

Radioligand and antiserum were then added, each in 0.1 ml PGB. Investigating two antisera and two radioligands, four RIA systems were explored: either 20,000 d.p.m. of E_3-16G-[^{125}I] or 10,000 d.p.m. of [^{3}H]-E_3-16G, dissolved in 0.1 ml of PGB, was added to each RIA tube as radioligand, followed by 0.1 ml of appropriately diluted anti-E_3-16G-2-ABA-BSA or anti-E_3-16G-BSA serum. Anti-E_3-16G-2-ABA-BSA serum was diluted 80-fold with PGB for use with either radioligand whereas anti-E_3-16G-BSA serum was diluted 1:320 in conjunction with [^{3}H]-E_3-16G and 1:1500 in combination with E_3-16G-[^{125}I]. The RIA tubes were vortexed and incubated for 16–18 h at 4°C.

Free and antibody-bound radioligands were separated by using 0.8 ml of a dextran-coated charcoal suspension (500 mg of charcoal and 50 mg of dextran T-70 in 100 ml of PGB) chilled at 4°C and by centrifugation at 4°C. The antibody-bound E_3-16G-[^{125}I] was counted in a Nuclear-Chicago Model 1085 γ-counter after decanting the supernatants from the charcoal pellets into 10 × 75 mm RIA tubes. When [^{3}H]-E_3-16G was employed as radioligand, 0.5 ml of the supernatant was transferred into counting vials and simultaneously dissolved in scintillation liquid by using a semiautomatic pipettor/dilutor (Labindustries, Berkeley, CA). Counting was carried out in a Nuclear-Chicago Mark II liquid scintillation spectrophotometer.

Logit transformation [12] was employed to construct standard curves and to calculate E_3-16G concentrations of unknown samples using a programmable Monroe model 1766 electronic calculator.

Sephadex gel filtration. In those experiments in which the need for purification of the E_3-16G fraction was explored, urine samples were subjected to gel filtration on a 1 × 50 cm Sephadex G-25 (medium) column [13]. The E_3-16G elution pattern had been determined with the aid of [^{3}H]-E_3-16G added to several urine specimens.

RESULTS

Reliability of estriol-16-glucuronide radioimmunoassays

The following radioligand/antibody systems were evaluated: No. 1: [^{3}H]-E_3-16G/anti-E_3-16G-BSA serum; No. 2: E_3-16G-[^{125}I]/anti-E_3-16G-BSA serum; No. 3: [^{3}H]-E_3-16G/anti-E_3-16G-2-ABA-BSA serum; and No. 4: E_3-16G-[^{125}I]/anti-E_3-16G-2-ABA-BSA serum. Of these four combinations, only the last system could not be utilized for a RIA of E_3-16G. Forty percent of the E_3-16G-[^{125}I] incubated with 0.1 ml of 80-fold diluted anti-E_3-16G-2-ABA-BSA serum in a total volume of 0.4 ml of PGB was bound to the antibody when no additional E_3-16G was added. However, logit B/B_0 vs. log dose E_3-16G standard curves were neither linear nor reproducible when the E_3-16G-[^{125}I]/anti-E_3-16G-2-ABA-BSA serum combination was used. The first three RIA systems, on the other hand, yielded reproducible standard curves depicted in Fig. 1.

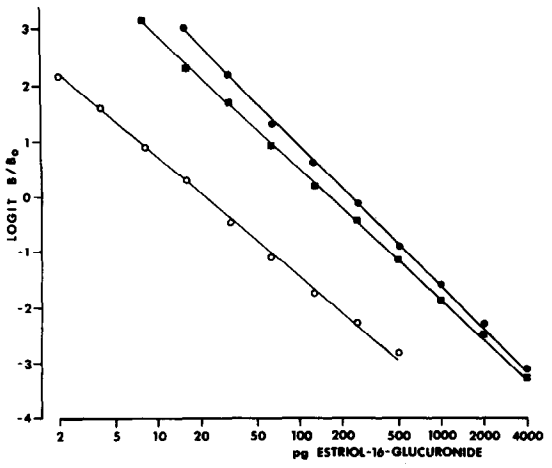

Fig. 1. Standard curves for the three radioimmunoassays compared in this study: (1) [^{3}H]-estriol-16-glucuronide/anti-estriol-16-glucuronide-bovine serum albumin serum (squares). (2) estriol-16-glucuronide-[^{125}I]-iodohistamine/anti-estriol-16-glucuronide-bovine serum albumin serum (circles) and (3) [^{3}H]-estriol-16-glucuronide/anti-estriol-16-glucuronide-2-azobenzoic acid-bovine serum albumin serum (dots).

Sensitivity

The linear range of the logit B/B_0 vs. log dose standard curves obtained with RIA systems No. 1–3 as well as the lowest detectable value which differs from zero by two standard deviations are shown in Table 1.

Accuracy

The accuracy of RIA's No. 1–3 was assessed as follows. Authentic E_3-16G was added to aliquots of a healthy child's urine which had been 10-fold diluted with PGB. The initial urine aliquot containing 20 ng/ml of E_3-16G was serially diluted with urine–PGB (1:10, V/V) to yield urines with E_3-16G concentrations which equalled those of serially diluted solutions of E_3-16G in PGB used to establish standard curves. Linear regression analysis of the E_3-16G measured in these aliquots (y), expressed in pg/ml and plotted vs. the amount of E_3-16G added to urine in pg/ml (x), yielded the equations listed in Table 1. The excellent coefficient of correlation of 0.999 was obtained in all three RIA's.

Precision

Intra- and inter-assay coefficients of variation were determined only for RIA No. 3 which was used to determine daily 24-h urinary E_3-16G excretion during 7 menstrual cycles: these were 4.3 and 9.3%, respectively.

Specificity

Cross-reactions of estriol and various estrogen conjugates in RIA systems No. 1–3 are listed in Table 2. The highest specificity was obtained with the [^{3}H]-E_3-16G/anti-E_3-16G-2-ABA-BSA serum RIA (system No. 3). Estriol (E_3) and estrone sulfate cross-reacted approx. 50 and 40%, respectively, in systems No. 1 and 2. The extent of these cross-reactions was quite similar for both the [^{3}H]-E_3-16G/anti-E_3-16G-BSA serum RIA (system No. 1) and the E_3-16G-[^{125}I]/anti-E_3-16G-BSA serum RIA (system No. 2).

Measurement of urinary estriol-16-glucuronide

Comparison of three different radioimmunoassays. E_3-16G was measured in 10 different urine aliquots collected from non-pregnant women, employing RIA systems No. 1–3. The data listed in Table 3 indicate that RIA's No. 1 and 2 using [^{3}H]-E_3-16G and E_3-16G-[^{125}I], respectively, in conjunction with anti-E_3-16G-BSA serum yielded comparable results, although in 8 of the 10 urines tested, slightly higher E_3-16G concentrations were obtained with system No. 2. The third RIA, however, utilizing [^{3}H]-E_3-16G in combination with anti-E_3-16G-2-ABA-BSA serum, rendered considerably lower E_3-16G concentrations in all 10 specimens examined. The results obtained by RIA No. 3 were, on the average, 26% lower than the mean of the values measured with RIA's No. 1 and 2, suggesting that system No. 3 may be the most specific one of these three RIA's.

Radioimmunoassay without and with preceding Sephadex G-25 gel filtration. In order to test whether Sephadex gel filtration of urine aliquots prior to RIA of the eluted E_3-16G fraction would enhance the

Table 1. Linear range of standard curves, sensitivity and accuracy of radioimmunoassays (RIA's) for estriol-16-glucuronide (E_3-16G) using [3H]-E_3-16G or estriol-16-glucuronide-[^{125}I]-iodohistamine (E_3-16G-[^{125}I]) as radioligand and anti-estriol-16-glucuronide-bovine serum albumin (anti-E_3-16G-BSA) serum or anti-estriol-16-glucuronide-2-azobenzoic acid-bovine serum albumin (anti-E_3-16G-2-ABA-BSA) serum as antibody

RIA No.	Radioligand/Antibody	Linear range of standard curve*	Sensitivity†	Accuracy‡
1	[^{3}H]-E_3-16G/anti-E_3-16G-BSA serum	8–4000 pg	8 pg	$y = 1.11\ x - 12.6$; $r = 0.999$
2	E_3-16G-[^{125}I]/anti-E_3-16G-BSA serum	2–500 pg	2 pg	$y = 0.99\ x + 1.5$; $r = 0.999$
3	[^{3}H]-E_3-16G/anti-E_3-16G-2-ABA-BSA serum	16–4000 pg	16 pg	$y = 1.13\ x - 9.9$; $r = 0.999$

* Logit B/B_0 vs log dose standard curves. † Defined as the smallest amount of E_3-16G per RIA tube which reduces the number of c.p.m. of labeled E_3-16G bound at zero mass by two standard deviations. ‡ y represents the E_3-16G measured in urine and x the amount of E_3-16G added to urine (both in pg/ml); r is the coefficient of correlation.

Table 2. Percent cross-reaction of unconjugated estriol and various estrogen conjugates with anti-estriol-16-glucuronide-bovine serum albumin (anti-E_3-16G-BSA) serum and anti-estriol-16-glucuronide-2-azobenzoic acid-bovine serum albumin (anti-E_3-16G-2-ABA-BSA) serum in conjunction with [^{3}H]-estriol-16-glucuronide ([^{3}H]-E_3-16G) or estriol-16-glucuronide-[^{125}I]-iodohistamine (E_3-16G-[^{125}I])

Steroids	Percent cross-reaction* Anti-E_3-16G-BSA serum [^{3}H]-E_3-16G	E_3-16G-[^{125}I]	Anti-E_3-16G-2-ABA-BSA serum [^{3}H]-E_3-16G
Estrone glucuronide	0.01	0.01	0.01
Estrone sulfate	37.5	44.1	0.01
Estradiol-3-glucuronide	0.01	0.01	0.01
Estradiol-17β-glucuronide	0.37	0.18	0.01
Estradiol-3-sulfate	0.01	0.02	0.6
Estriol	48.3	51.3	2.1
Estriol-3-glucuronide	0.01	0.06	0.01
Estriol-16-glucuronide	100	100	100

* Defined according to Thorneycroft *et al.*[14] as $(x/y) \times 100$ where x is the mass of estriol-16-glucuronide and y the mass of the heterologous steroid at which the binding of [^{3}H]-estriol-16-glucuronide or estriol-16-glucuronide-[^{125}I]-iodohistamine is reduced by 50%.

Table 3. Measurement of urinary estriol-16-glucuronide concentrations (ng/ml) in 10 different urine samples by 3 radioimmunoassay (RIA) systems: RIA No. 1: [^{3}H]-estriol-16-glucuronide/anti-estriol-16-glucuronide-bovine serum albumin serum; RIA No. 2: estriol-16-glucuronide-[^{125}I]-iodohistamine/anti-estriol-16-glucuronide-bovine serum albumin serum; and RIA No. 3: [^{3}H]-estriol-16-glucuronide/anti-estriol-16-glucuronide-2-azobenzoic acid-bovine serum albumin serum

Urine sample	Urinary estriol-16-glucuronide concentration (ng/ml) RIA No. 1	RIA No. 2	RIA No. 3
1	4.41	4.66	3.17
2	6.24	5.65	4.45
3	10.4	10.4	6.98
4	14.3	17.4	11.5
5	12.0	13.1	8.36
6	12.6	12.7	8.93
7	13.1	14.2	10.6
8	6.98	7.30	5.42
9	4.53	4.70	3.61
10	38.4	41.1	33.8

Table 4. Urinary estriol-16-glucuronide concentrations (ng/ml) in 10 different urine aliquots without and with preceding gel filtration on a 1 × 50 cm Sephadex G-25 column. The RIA employed [^{3}H]-estriol-16-glucuronide and anti-estriol-16-glucuronide-2-azobenzoic acid-bovine serum albumin serum

Urine sample	Urinary estriol-16-glucuronide concentration (ng/ml) Without gel filtration	Following gel filtration
1	2.53	2.06
2	2.60	2.24
3	2.78	2.02
4	4.78	3.86
5	4.01	3.55
6	6.37	6.62
7	9.47	9.78
8	7.61	7.33
9	7.46	6.44
10	8.63	8.04

specificity of the RIA, E_3-16G was measured in 10 urine aliquots obtained from 10 non-pregnant women with and without preceding gel filtration on Sephadex G-25 columns. The results listed in Table 4 indicate that, on the average, E_3-16G concentrations measured by the most specific RIA ([^{3}H]-E_3-16G/anti-E_3-16G-2-ABA-BSA serum) after Sephadex gel filtration were 11% lower than those obtained by the same RIA system without preceding purification.

Radioimmunoassay of daily 24-h urinary estriol-16-glucuronide excretion. E_3-16G was determined by direct RIA using [^{3}H]-E_3-16G and anti-E_3-16G-2-ABA-BSA serum in 24-h urines collected by 7 volunteers daily throughout an entire menstrual cycle. Individual serum E_2 and urinary E_3-16G results obtained in these 7 women were grouped according to the day of the midcycle LH peak and averaged. These daily

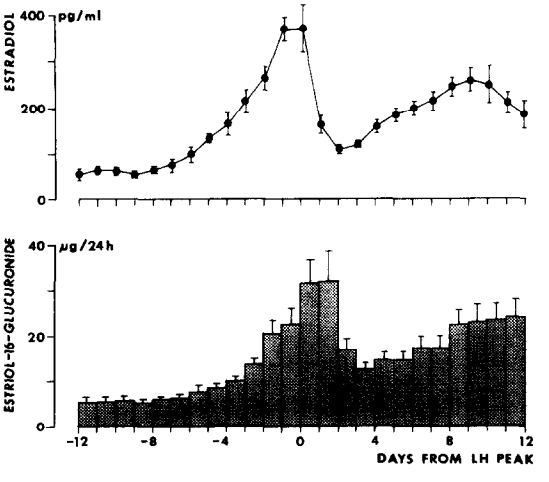

Fig. 2. Means and standard errors of serum estradiol concentrations measured each morning at 8 a.m. and daily 24-h urinary estriol-16-glucuronide excretion in 7 women during a menstrual cycle. The data obtained in individual subjects were grouped according to the day of the midcycle LH peak and averaged.

means of serum E_2 and urinary E_3-16G values as depicted in Fig. 2 demonstrate good correlation.

DISCUSSION

Employing the method described by Nars and Hunter[10] for the synthesis of estradiol-6-(O-carboxymethyl)oximino-[^{125}I]-iodohistamine, we succeeded in preparing E_3-16G-[^{125}I] to be used as a radioligand for the RIA of E_3-16G. Although E_3-16G-[^{125}I] bound to both anti-E_3-16G-BSA and anti-E_3-16G-2-ABA-BSA serum when no authentic E_3-16G was added (at 0 mass), a reproducible E_3-16G standard curve could only be obtained when the E_3-16G-[^{125}I] was employed in conjunction with the less specific anti-E_3-16G-BSA serum. The reason for failure to obtain acceptable standard curves with the E_3-16G-[^{125}I]/anti-E_2-16G-2-ABA-BSA serum combination remains enigmatic.

Three RIA systems with good standard curves for E_3-16G were compared: No. 1: [^{3}H]-E_3-16G/anti-E_3-16G-BSA serum; No. 2: E_3-16G-[^{125}I]/anti-E_3-16G-BSA serum; and No. 3: [^{3}H]-E_3-16G/anti-E_3-16G-2-ABA-BSA serum. All three RIA's were accurate and precise. Assay sensitivity and specificity, however, differed between these three RIA's. Most sensitive was RIA No. 2 and least sensitive was RIA No. 3. RIA No. 1 which employs [^{3}H]-E_3-16G was only one-fourth as sensitive as RIA No. 2 using E_3-16G-[^{125}I] in conjunction with the same anti-E_3-16G-BSA serum as RIA No. 1. Thus utilizing the [^{125}I]-labeled, instead of the tritiated, radioligand enhanced the sensitivity of the RIA 4-fold.

Both RIA's No. 1 and 2 were less specific than RIA No. 3 which utilizes the highly specific anti-E_3-16G-2-ABA-BSA serum [2]. Unconjugated E_3 and estrone sulfate exhibited large and comparable cross-reactions in RIA's No. 1 and 2, averaging 50 and 41%, respectively. It was therefore not surprising that RIA No. 3 yielded 26% lower urinary E_3-16G results than did RIA's No. 1 and 2. Although the results obtained by RIA No. 1 and 2 were comparable, the E_3-16G concentrations measured by RIA No. 2 were, similar to the cross-reactions of estriol and estrogen conjugates seen in RIA No. 2, slightly larger than those observed by RIA No. 1. Sephadex G-25 gel filtration reduced the E_3-16G measurement in RIA No. 3, the most specific RIA system tested in this study, by only 11%. It was felt that the mere 11% reduction of cross-reacting contaminant(s) would not justify the relatively tedious Sephadex gel filtration process. Thus, E_3-16G was measured in this study in unextracted 10-fold diluted urine without a preceding purification step.

Evaluation of the reliability criteria of RIA's No. 1–3 revealed that the [^{3}H]-E_3-16G/anti-E_3-16G-2-ABA-BSA serum system (No. 3) was the most specific, although the least sensitive, RIA for E_3-16G determinations. Because its sensitivity sufficed for the intended purpose and its accuracy and precision were as high as those of the other two methods, RIA No. 3 was chosen to measure the daily 24-h urinary E_3-16G excretion in 7 women throughout an entire menstrual cycle. The results obtained in this study compare favorably with those observed by colorimetric [15] and fluorimetric [16] determinations of total urinary E_3 excretion. The E_3-16G excretion pattern mimicks that of serum E_2 concentrations observed in the same 7 women. In Fig. 2 where both the individual daily serum E_2 concentrations and the 24-h urinary E_3-16G values of the 7 women are grouped according to the midcycle LH peak and averaged, the steep ascending slope of the preovulatory serum E_2 surge is followed by rapidly rising E_3-16G excretion. However, the preovulatory E_3-16G peak occurs 1 to 2 days later than the corresponding serum E_2 surge. The delay in urinary E_3-16G excretion is explained by the temporal relationships of E_2 and E_3 metabolism, conjugation and excretion [16–20].

A significant rise in urinary E_3-16G excretion (i.e. values exceeding two standard deviations of the baseline 24-h urinary E_3-16G values during the midfollicular phase) was observed 2, 3, 4 and 5 days prior to the morning of the midcycle LH peak in 2, 1, 2 and 2 of the 7 women studied, respectively. This distinct preovulatory rise of urinary E_3-16G confirms the findings of Lehtinen and Adlercreutz [5] who reported a rise in urinary E_3-16G concentrations 4–7 days prior to ovulation in 3 subjects studied. Thus, serial E_3-16G determinations, rapidly and conveniently performed by this direct RIA technique, appear to be useful for the prediction of ovulation and for gauging ovarian response to exogenous gonadotropins given to induce ovulation. However, when urinary estradiol-17β-glucuronide (E_2-17G) excretion was measured in these 7 volunteers, a significant rise of E_2-17G excretion preceded that of E_3-16G by 1 to 5 days in 6 of the 7 study subjects [21]. Thus for the prediction of ovulation, urinary E_2-17G, which as a metabolite of E_2 is excreted more rapidly than E_3-16G, may be a more desirable test substance than urinary E_3-16G.

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