AN ASSAY FOR URINARY ESTRIOL-16α-GLUCURONIDE BASED ON ANTIBODY-ENHANCED CHEMILUMINESCENCE

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ABSTRACT

An immunoassay for estriol-16α-glucuronide in pregnancy urine is described that utilizes antibody-enhanced chemiluminescence. The steroid glucuronide was covalently conjugated with the chemiluminescent marker aminobutyl-ethyl-isoluminol. The light yield of this conjugate upon oxidation was augmented by specific antibody, and this effect was inhibited by addition of the homologous steroid glucuronide (10-100 pg) in a dose-dependent manner. The assay does not require separation of bound and free ligand and proved satisfactory with respect to sensitivity, precision and accuracy. Assay results obtained by radioimmunoassay and chemiluminescence immunoassay were in good agreement ($r = 0.98$; $n = 25$).

INTRODUCTION

It has been shown that fetal well-being can be assessed by measurement of urinary estriol-16α-glucuronide [1] using radioimmunoassay (RIA) methods [2,3]. Existing RIA procedures have the advantage of extreme sensitivity, but depend on the availability of a suitable radiolabeled ligand and of expensive equipment. Furthermore, RIA procedures require a time-consuming phase-separation step and disposal of radioactive waste has become a serious problem.

Several approaches have been described to overcome these drawbacks of RIA [4]. Some of these methods use fluorescence [5] or chemiluminescence [6-8] as an end point and do not require separation of bound and free hormone. We explored the possibility of developing immuno-

assays based on antibody-enhanced chemiluminescence for urinary steroid glucuronides, since chemiluminescent labels can be detected at pM levels [9], and determined in less than 10 seconds.

The method described here follows an approach used earlier for biotin [6,7,9] and for progesterone [8]. The carboxy group of the steroid-glucuronide was attached covalently to the free amino group of the chemiluminescent marker aminobutyl ethyl isoluminol. The resulting steroid glucuronide-chemiluminescent marker conjugate emits light upon oxidation with microperoxidase and H_2O_2. When the steroid glucuronide chemiluminescent marker conjugate is bound to specific binding protein, the total light production of the conjugate is enhanced. This binding and the consequent enhancement of light emission is inhibited in a competitive manner by the addition of unaltered glucuronide.

MATERIALS AND METHODS

Reagents. Estriol-16α-glucuronide (20 mg) was obtained through the courtesy of Dr. W.F. Coulson of the Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London. Microperoxidase (MP-11), N,N^1 -dicyclohexyl-carbodiimide, N-hydroxysuccinimide and bovine serum albumin (Fraction V) (BSA) were purchased from Sigma, St. Louis, Mo.; Sepharose-Protein A and prepacked disposable PD-10 columns Sephadex G-25 M from Pharmacia, Uppsala, Sweden; 30% hydrogen peroxide solution and precoated silica gel G60 thin layer chromatography plates from Merck, FRG; and XAD-2 non-ionic resin from Serva, Heidelberg.

Antiserum (0.5 ml) to estriol-16α-glucuronide and 250 μCi of tritiated estriol-16α-glucuronide (40 Ci/mmol) were received from Dr. W.F. Coulson.

Anti-estriol-16α-glucuronide IgG was prepared by chromatography on a protein A Sepharose CL-4B affinity column as follows. The antiserum was dialyzed against 0.1 M sodium phosphate (pH 7), and the retentate derived from 0.5 ml serum was applied to the affinity column (1 ml of packed gel). The immunoglobulin fraction was eluted with 10% acetic acid, neutralized immediately to pH 7, dialyzed against 0.05 M sodium phosphate (pH 8), divided into 0.5 ml lots and stored at -20°C. The titer and affinity of anti-estriol-16α-glucuronide IgG fraction were determined by radioimmunoassay procedures, using 0.05 M sodium phosphate (pH 8) containing 0.1 M NaCl, 0.1% NaN_3 and 0.1% BSA, as the assay buffer. The amount of specific antibody in 1% IgG solution, determined

according to [10], was 1.78 mg/ml. The affinity constant (K_a) was 3.3×10^{10} liter/mol. The assay buffer in the chemiluminescent reactions was 0.05 M sodium phosphate (pH 8.0) containing 6 g NaCl/liter.

Stock solutions of steroids and chemiluminescent compounds were prepared in ethanol and in water respectively. They were stored at 4°C and diluted to the desired concentration in assay buffer when required.

Microperoxidase was dissolved at 1 mg/ml in 0.01 Tris-HCl (pH 7.4); this stock solution was kept at 4°C. The working solution was obtained by diluting the stock solution in assay buffer to 2.6 µM enzyme. The oxidant solution was prepared by adding 30 µl of 30% H_2O_2 solution to 5 ml borate buffer (pH 8.6; 0.06 M).

Synthesis of estriol-16α-glucuronide ABEI conjugate. This conjugate was synthesized in two steps.

1. Estriol-16α-glucuronide (6 mg) was dissolved in dry dimethylformamide (0.2 ml) and equivalent amounts of N,N'-dicyclohexyl-carbodiimide (2.4 mg) and N-hydroxysuccinimide (1.5 mg) were added to the solution. The eluate containing the activated N-succinimide ester of estriol-16α-glucuronide was used in the next step without further purification.

2. 6[N-(6-aminobutyl)-N-ethyl]-amino-2,3-dihydrophthalazine-1,4-dione (ABEI) (prepared according to Schroeder et al., ref. 7) (3.5 mg) and 0.2 ml of 0.13 M $NaHCO_3$ solution were added to the dimethylformamide solution containing the activated ester. The mixture was stirred for 4 hours, neutralized to pH 6 and chromatographed on PD-10 Sephadex G-25 M column, using water as the eluant. Fifteen 0.5 ml fractions were collected. The purity of each fraction was examined by thin layer chromatography using chloroform-methanol (60:40) as the developing solvent. Fraction number six and seven showed only one fluorescent spot with R_f = 0.76. In this system ABEI and estriol-16α-glucuronide had R_f 0.06 and 0.16 respectively. The U.V. spectrum of fraction six and seven (in water) showed peaks at 270 nm, 285 nm and 320 nm, coinciding with peaks present in the spectra of the aminopentyl ethyl isoluminol derivatives of free estradiol and estriol (data not shown). It was concluded that these fractions contained estriol-16α-glucuronide-ABEI conjugate (Fig. 1), and the concentration of the conjugate was determined by using ε = 18,000 at 285 nm. Estriol-16α-glucuronide-ABEI conjugate showed the same affinity as unaltered estriol-16α-glucuronide when examined for its ability to compete with tritiated estriol-16α-glucuronide for the binding sites of anti-estriol-16α-glucuronide IgG.

Light measurements. Measurements of light emission were made with a Lumac Luminometer Model 2080 (Lumac Systems, Basel) using the automatic injection and integration modes of the instrument, and Lumacuvette P polystyrene test tubes (12 x 50 mm) as reaction vessel. The Luminometer was also connected to a storage oscilloscope (Type 5111, Tektronix, Beaverton, Oregon) in order to observe the kinetics of light emission. When the Luminometer was used in the automatic injection mode, readings on the Luminometer started two seconds after initiation of the light reaction.

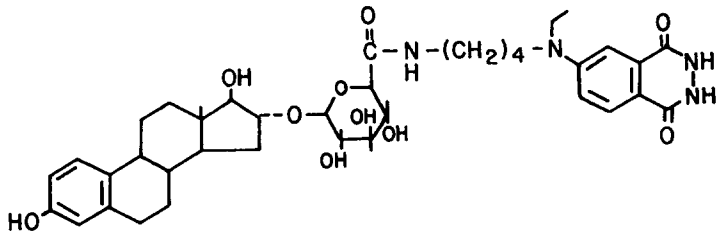

Fig. 1: Proposed structure for estriol-16α-glucuronide aminobutyl ethyl isoluminol (ABEI) conjugate.

Since the peak intensity of the light emission occurred within one sec, as indicated by the oscilloscope, the readings recorded by the Luminometer corresponded to the decay part (DP) of the chemiluminescent reaction. The light emission was routinely measured over the 10 sec interval from the end of 2nd to the 12th sec after adding the oxidant, and recorded as arbitrary light units. This quantity will be referred to as the DP light yield.

Collection of samples. Twenty-four h urine samples were collected without preservative from pregnant women (21-41 weeks of gestation) and from post-partum women and aliquots were stored at -20°C until analysis.

Sample preparation. The urine samples for the assay were first adsorbed on columns containing Amberlite XAD-2 resin [11]. The columns were washed with assay buffer and the steroid glucuronide was then eluted with methanol. The following procedure was adopted for exploratory work in our laboratory: Stir 10 g of XAD-2 resin with 50 ml of methanol. Decant the methanol layer in order to remove the fines. Repeat the methanol washing; then wash the resin several times with buffer. Make a slurry of the resin with 50 ml of buffer. Pack 1.2 x 6 cm plastic columns containing glass fiber plugs at the bottom with 4.0 ml of Amberlite resin. Pass 0.2 ml of urine specimen containing 3000 cpm of (^{3}H)-estriol-16α-glucuronide through the column. Wash the column with 25 ml of buffer. Elute the steroid glucuronide with 4.0 ml of methanol. Transfer a portion of this solution (1 ml) to a counting vial to assess recovery. Take an aliquot (0.1 ml) of the remainder, dilute with assay buffer 20- to 100-fold and use this diluted methanolic eluate for the chemiluminescence immunoassay. The addition of internal radioactive standard was later discontinued, since preliminary experiments indicated that recovery of radioligand from urine using the procedure outlined above was consistently greater than 90%.

Assay procedure. Dose response curves were established at the beginning and end of the assay. The unknown samples were run in duplicate and at two different dilutions. The assay was assembled in the following manner, using Lumacuvettes: standards (10-120 pg steroid glucuronide) or urine extracts prepared as described in the preceeding paragraph dissolved in 0.1 ml assay buffer were added to the appropriate tubes (see Table 1). An enzyme/oxidant blank tube, a basal chemiluminescence control tube and a maximal antibody-enhancement tube were set up as indicated in Table 1. Specific antibody (0.1 ml solution containing 0.12 pmol IgG/tube) was added to all tubes except the enzyme/oxidant blank and basal control tubes, which received 0.1 ml assay buffer instead. All tubes were incubated at 4°C for 30 min. Steroid glucuronide chemiluminescent marker conjugate (0.1 ml) was then added to all tubes except enzyme/oxidant blank tubes. All tubes were incubated for 30 min at 4°C; enzyme solution (0.1 ml) was added; oxidant (0.1 ml) was injected to each tube and light emission was measured for 10 sec.

TABLE 1: Lay-out of the chemiluminescence immunoassay.

	Designation of tube				
	Enzyme/Oxidant Blank	Basal Control	Maximal Enhancement	Standard	Unknown
Reagent added:					
E_3-16α-glucuronide	-	-	-	+	-
or					
Urinary extract	-	-	-	-	+
E_3-16α-glucuronide ABEI conjugate	-	+	+	+	+
Specific anti-E_3-IgG	-	-	+	+	+
Enzyme solution	+	+	+	+	+
H_2O_2 solution	+	+	+	+	+

Calculations. Luminometer readings were plotted against concentrations of estriol-16α-glucuronide (cf. Fig. 5). The amount (pg) of estriol-16α-glucuronide present in each assay tube was read directly from this curve and was converted to the appropriate unit (e.g. nmol/liter urine) by taking into consideration the volume of urine extracted and the dilution used. Cross reactivity (%) was calculated as proposed by Thorneycroft, et al., [12] as $\frac{100 \times}{y}$ where x is the mass of estriol-16α-glucuronide and y the mass of heterologous compound required to produce 50% inhibition of the binding of estriol-16α-glucuronide ABEI conjugate by antibody.

RESULTS

Light-yield of estriol-16α-glucuronide-ABEI conjugate. Conditions were first sought that would reduce to a minimum the light signal obtained upon addition of hydrogen peroxide to a solution containing the enzyme only. This was attained by using 100 µl of a 2.6 µM enzyme solution and 100 µl of 0.19% H_2O_2 solution in borate buffer, pH 8.6. Using these conditions, the DP light yield (see light measurements in Methods section) viz., the light emitted during the 3rd to 12th sec after addition of the oxidant, increased linearly with estriol-16α-glucuronide ABEI concentration (Fig. 3, lower curve) and the lower limit of detection (2 SD above the enzyme oxidant blank value) was 4 pg/tube.

Effect of anti-estriol-16α-glucuronide IgG on the light yield during oxidation of estriol-16α-glucuronide-ABEI conjugate. When varying amounts of the γ-immunoglobulin fraction (IgG) of an antiserum to estriol-16α-glucuronide BSA were incubated with 25 pg of estriol-16α-glucuronide-ABEI conjugate, the light yield upon oxidation increased as a function of IgG concentration up to about 1.5 pmol IgG/tube (Fig. 2). Normal IgG or heterologous IgG, e.g., IgG from anti-cortisol sera, did not enhance light production (data not shown).

In additional experiments, varying levels of estriol-16α-glucuronide were incubated with or without a constant amount of anti-estriol-16α-glucuronide IgG (0.12 pmol/tube) for 1 h at 4°C (Fig. 3). In the absence of anti-estriol-16α-glucuronide IgG, the light yield produced by oxidation of estriol-16α-glucuronide IgG increased linearly with the concentration of estriol-16α-glucuronide-ABEI conjugate up to 500 pg/tube. When anti-estriol-16α-glucuronide IgG (0.12 pmol/tube) was added, the light yield increased linearly with the concentration of the

conjugate up to 120 pg/tube. The antibody-induced enhancement over this range was 7-fold (Fig. 3).

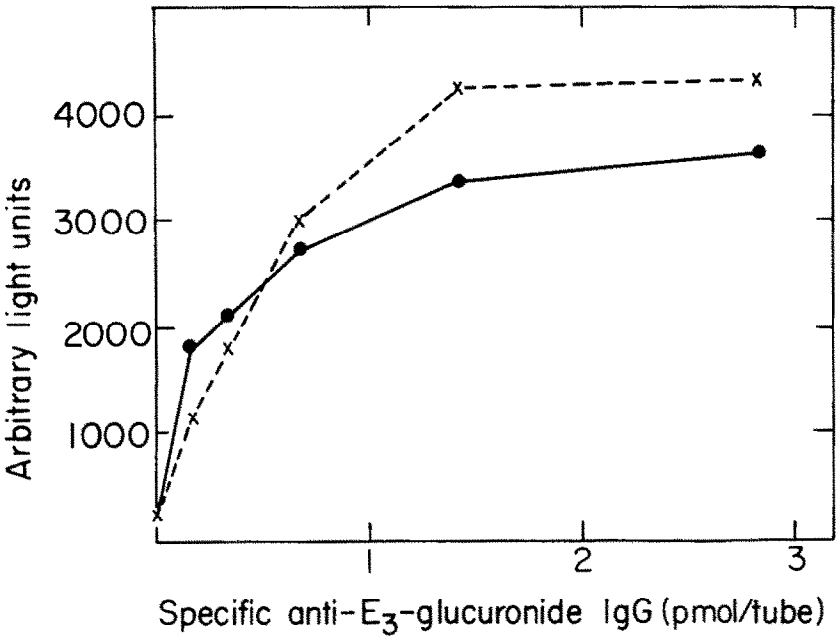

Fig. 2: Effect of anti-estriol-16α-glucuronide IgG concentration on the light yield produced by estriol-16α-glucuronide-ABEI conjugate upon oxidation. Varying levels of anti-estriol-16α-glucuronide IgG were incubated at 4°C for 10 sec (x----x) or 30 min (●——●) with 25 pg of estriol-16α-glucuronide-ABEI conjugate in a total volume of 300 µl of assay buffer. Microperoxidase (100 µl of a 2.6 µM solution) was added; the reaction tube was then introduced into the Luminometer and 100 µl of the oxidant (0.19% of H_2O_2 solution in borate buffer, pH 8.6, 0.06 M) was injected. Light emission was integrated over the period 3-12 sec after adding the last reagent and is expressed in arbitrary units.

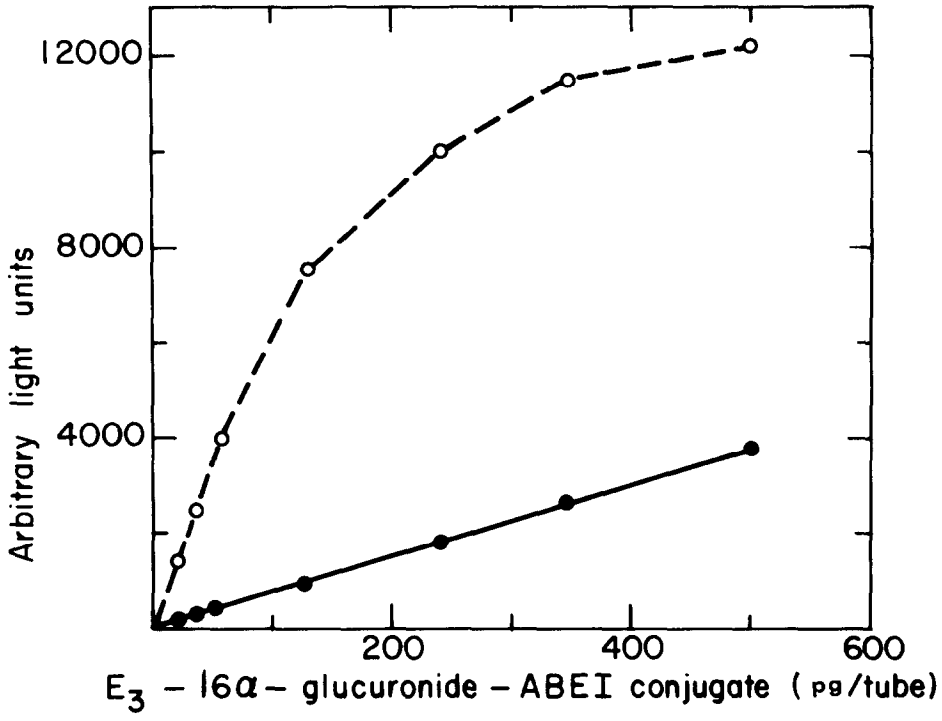

Fig. 3: Effect of estriol-16α-glucuronide conjugate concentration on IgG-enhanced light emission. Reaction mixtures containing various levels of estriol-16α-glucuronide-ABEI conjugate in 300 µl assay buffer were incubated at 4°C for 1 h with (o) or without (•) 0.12 pmol anti-estriol-16α-glucuronide IgG. Further treatment as described in legend to Fig. 2.

Development of an assay procedure for urinary estriol-16α-glucuronide based on antibody-enhanced chemiluminescence

Removal of interfering luminescent compounds in urine. When diluted male urine was mixed with the enzyme solution and the oxidant in the absence of a steroid-glucuronide chemiluminescent marker conjugate, a light signal was obtained (Fig. 4). Although the chemiluminescent effect of urine decreased with increasing dilution of urine, a preliminary purification of diluted urine was necessary in order to determine

reliably the steroid glucuronide. This was accomplished by adsorbing diluted urine on non-ionic Amberlite XAD-2 resin. Most of the interfering luminescent substances were eliminated in a buffer wash and the labeled steroid was recovered in the methanolic eluate. Thus, when 1:10 diluted male urine containing tritiated estriol-16α-glucuronide was chromatographed on the resin, the recovery of the radiolabeled steroid from the resin was 92.3 ± 5.2%. The light-yield of a male urine extract thus prepared did not significantly exceed that of the enzyme-oxidant blank (<4 pg steroid equivalent).

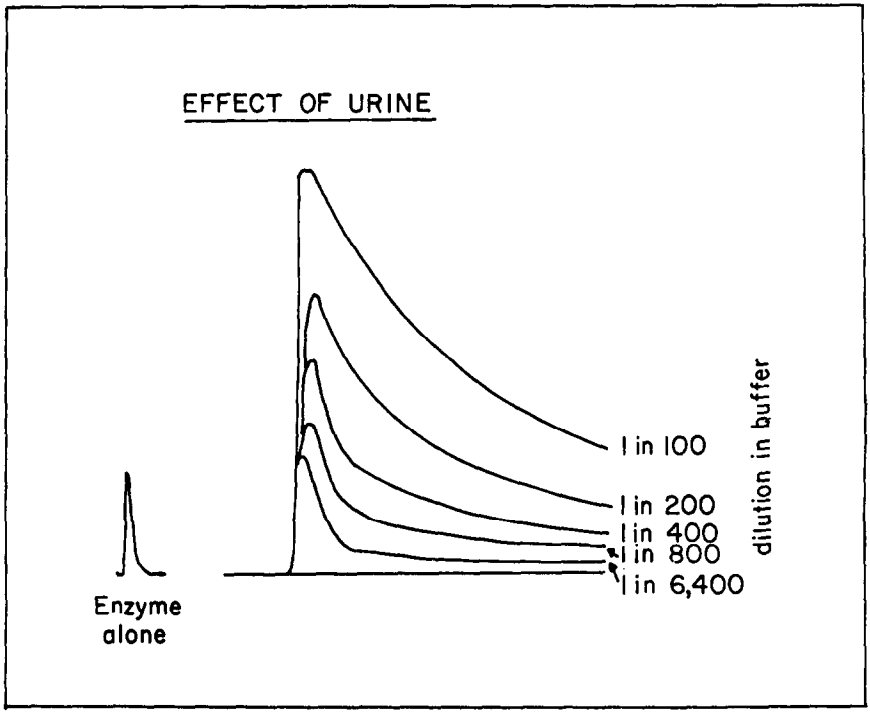

Fig. 4: Chemiluminescent effect of urine as a function of dilution. Male urine was diluted serially with assay buffer and mixed with the oxidizing reagents in the absence of a steroid-glucuronide-chemiluminescent marker conjugate. The oscilloscope tracing obtained for each dilution is shown. Speed: 2 sec/1.22 cm; sensitivity: 100 mV/1.22 cm.

Validation of the estriol-16α-glucuronide immunoassay

Specificity of the assay. The specificity of the immunoassay using anti-estriol-16α-glucuronide IgG and chemiluminescence was similar to that observed when using the same antiserum in a radioimmunoassay: light emission was not significantly affected by addition of cortisol, progesterone, testosterone, estrone-3-glucuronide or estradiol-3-glucuronide; cross reaction (see Methods for definition) was 20% for estrone, 25% for estradiol and 48% for estriol.

Sensitivity of the assay. Competitive binding reactions were carried out by incubating varying amounts of free estriol-16α-glucuronide with a constant amount of anti-estriol-16α-glucuronide as described above (See Assay procedure in Methods Section). The DP light yield (see light measurements) measured over 10 sec decreased with increasing estriol-16α-glucuronide over the 10-100 pg/assay tube. Figure 5 shows a representative dose response curve obtained from the readings of the Luminometer. The least amount of unlabeled hormone that could be distinguished from zero ($p<0.05$) was 10 pg/tube.

Accuracy of the assay. As a measure of accuracy the recovery of unlabeled estriol-16α-glucuronide (20-80 µg/ml) added to 1 ml of male urine was determined using the procedure of sample preparation and chemiluminescence assay described in Materials and Methods. The results are shown in Table 2.

TABLE 2: Recovery of estriol-16α-glucuronide from urine

Added (μg/ml)	% Recovery
20	76.8 ± 4.5 (n = 4)
40	85.0 ± 2.3 (n = 4)
80	87.5 ± 8.2 (n = 4)

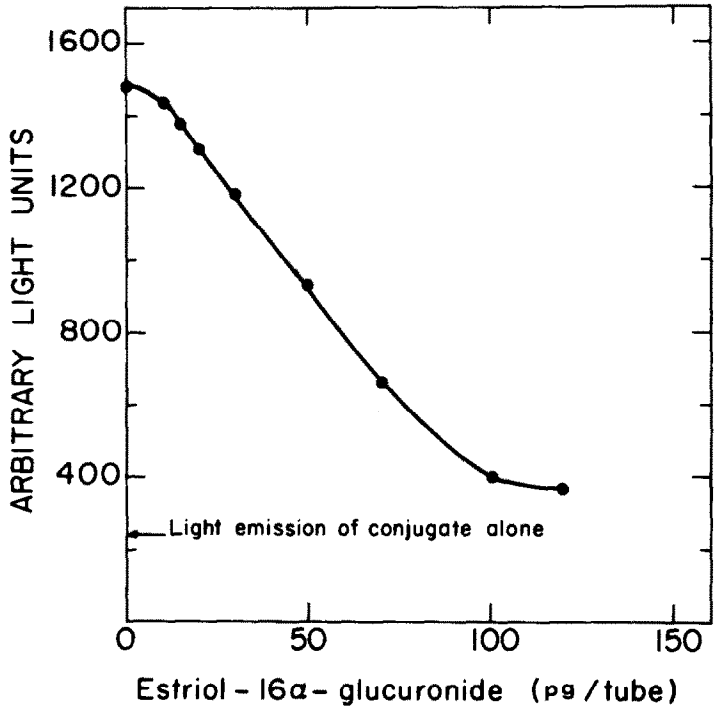

Fig. 5: Representative dose-response curve for estriol-16α-glucuronide measured by chemiluminescence immunoassay. Varying amounts of estriol-16α-glucuronide were incubated with 0.12 pmol of specific anti-estriol-16α-glucuronide IgG in a total volume of 200 μl of assay buffer for 30 min at 4°C. The estriol-16α-glucuronide-ABEI conjugate (20 pg in 100 μl of assay buffer) was then added, and the incubation was continued for another 30 min at 4°C. The enzyme solution (100 μl) was added, and the oxidant was injected in the dark. The ordinate shows the Luminometer readings for each concentration of estriol-16α-glucuronide.

Within-assay precision. The within-assay precision was calculated from the results of repeated analyses of two urine samples in the same assay. The results are shown in Table 3.

TABLE 3: Intra-assay variation for the urinary estriol-16α-glucuronide assay in pregnancy.

Sample No.	Mean ± S.D. (μg/ml)	Coefficient of variation	n	Dilution of urine extract used in the assay
33	10.97 ± 0.18	1.67	6	1:500
38	142.27 ± 2.88	2.02	5	1:4000
	149.96 ± 10.87	7.25	4	1:6000

Between-assay variation. The between-assay variation was calculated from the results of repeated analyses of five urine samples in a number of assays conducted on different occasions. The results are shown in Table 4.

TABLE 4: Between-assay variation for the urinary estriol-16α-glucuronide assay in pregnancy.

Sample No.	Number of assays	Mean ± S.D. (μg/ml)	Coefficient of variation
21	3	32.2 ± 5.14	15.9
22	3	22.06 ± 1.79	8.1
24	3	13.5 ± 0.43	3.2
35	3	33.46 ± 1.74	5.2
40	3	5.26 ± 1.06	20.3

Comparison of chemiluminescence immunoassay for urinary estriol-16α-glucuronide with radioimmunoassay. Urine samples of second and third trimester pregnancy (n = 15) and post-partum urine samples (n = 10) were processed as described in Materials and Methods. The eluates were assayed for estriol-16α-glucuronide by chemiluminescence

immunoassay and by radioimmunoassay, using the same antiserum. The results of the two methods agreed well ($r = 0.98$; $y = 1.15x - 2.94$), where y are the values determined by radioimmunoassay and x values determined by chemiluminescence.

DISCUSSION

This paper describes the development of an immunoassay for urinary estriol-16α-glucuronide based on antibody-enhanced chemiluminescence. This technique does not require physical separation of bound and free ligand, or counting of radioactivity. The sensitivity achieved (10 pg/tube) is comparable to that obtained by radioimmunoassay. Furthermore, the steroid-chemiluminescent marker conjugate is stable and can be stored at 4°C for at least a year without lost of activity. The entire assay, including the extraction step, is readily performed in two working hours, and the end point determination requires only 10 sec. Although at this stage recording of the light production was made manually, the lack of a phase-separation step makes the assay readily amenable to automation. The method eliminates the isotope disposal problems inherent in RIA. These features, and the ability to yield rapid results, make make the method attractive for clinical use. Although the instrumentation required for chemiluminescence immunoassay is less complex than that needed for RIA, the requisite apparatus is not, at this time, available in most hospital laboratories. However, it should be pointed out that these are pilot studies and the new methodology still requires validation using wider range of normal and biological material. Also, while a close correlation was observed between assay results obtained by chemiluminescence assay and the better established RIA procedures for urine samples from different patients ($r = 0.98$), we have no ready

explanation for the regression of coefficient from unit. Furthermore, the between assay coefficient of variation was rather high and was not consistently low; (range 3.2 - 20.3), a reflection, perhaps, of the sensitivity of the chemiluminescent reaction to factors that still require definition and closer control. Recognition of these factors should evolve further experience with the method. The present recovery rate of the sample preparation, using column chromatography, may also be capable of further improvement.

In spite of the present shortcomings of this method, the potential advantages of this novel approach to steroid immunoassay appear sufficiently encouraging to warrant further exploration.

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ABBREVIATIONS

E_3 = estriol