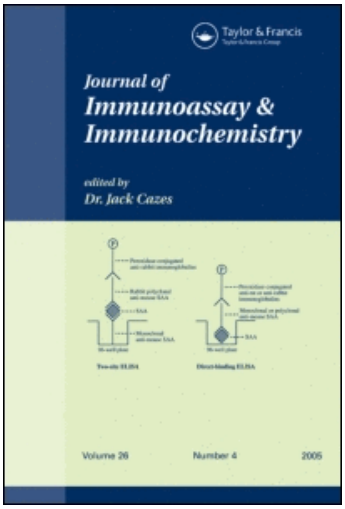

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Antigen Heterologous Enzyme Linked Immunosorbent Assay for the Measurement of Estrone-3-Glucuronide

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Antigen Heterologous Enzyme Linked Immunosorbent Assay for the Measurement of Estrone-3-Glucuronide

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Abstract: We report a novel antigen heterologous enzyme linked immunosorbent assay for the direct estimation of estrone-3-glucuronide (E1-3-G) in diluted human urine. The differential behavior of antibody towards the labeled and unlabelled analyte in heterologous systems forms the basis of the present assay. Antiserum was raised in New Zealand white rabbits using estrone-3-glucuronide-bovine serum albumin (E1-3-G-BSA) as immunogen and nandrolone-17-HS (N-17-HS) coupled to horseradish peroxidase (HRP) with and without urea bridge ((N-17-HS-Urea-HRP/N-17-HS-HRP) as an enzyme labeled reagent. To the E1-3-G antibody coated wells, 50 μL standard or appropriately diluted (1:20) female urine samples were added along with nandrolone-17-HS-HRP/N-17-HS-Urea-HRP conjugate (100 μL) and were incubated at room temperature (RT) for 1 hour. Bound enzyme activity was measured by using tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2) as substrate. The cross-reaction of E1-3-G antiserum with C_{18}, C_{19}, C_{21}, C_{27} steroids was less than 0.1% in both assays. Incorporation of urea bridge in the enzyme conjugate has decreased the effective displacement dose, i.e., ED_{50} from 20 ng/ml to as low as 2 ng/mL. The sensitivity of the assay using N-17-HS-HRP and N-17-HS-Urea-HRP was 0.6 ng/mL and 0.4 ng/mL, respectively. The intra-assay and inter-assay coefficient of variation (CVs) ranged from 4.7–9.0% and 5.1–6.4%, respectively. The estrone glucuronide level was also determined in female urine samples of 26 and 28 days cycle depicting a prominent peak corresponding to the

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preovulatory phase. The urinary E1-3-G values correlated well with those obtained by heat denaturation of urine samples r = 0.94 (n = 27).

Keywords: Estrone-3-glucuronide, Antigen heterologous, Nandrolone-17-HS, Urea bridge, ELISA

INTRODUCTION

Considerable attention has been directed towards determining the status of reproductive hormones owing to their physiological significance.[1–3] Estrone-3-glucuronide (E1-3-G) is a metabolite of estradiol, a primary steroid that regulates the reproductive function in human females. The measurement of E1-3-G in urine is preferred over estradiol to assess various aspects of ovarian function, since the urinary concentration of E1-3-G is higher than the corresponding serum concentration.

This allows the measurement of the lower end of the physiological range in humans. Also, the estimation of E1-3-G in urine samples scores over the use of other matrices like saliva, serum, and blood spots, in its easy availability in sufficient volume over the period of time; high patient compliance and samples can be self collected and stored by the patient. Furthermore, urine provides integrated hormone measures without the confounding effects of pulsatile secretion.[4]

Estrone-3-glucuronide holds immense importance in precisely predicting the impending ovulation and, hence, the duration of the fertile period. The prediction of delineation of the fertile period is an approach to natural family planning and is also eminent for donor insemination and in vitro fertilization.[5] The reasonable preovulatory surge and 100-fold permissible dilution of urine samples makes E1-3-G a suitable choice for the purpose.[6] Thus, over the period of time, various radioimmunoassay procedures for E1-3-G measurement have been developed[7–9] which were later replaced by non-isotopic methods.[10–14] While the introduction of these non-isotopic assays overcomes the cost and radiation hazard of RIAs, the lack of higher specificity, along with good sensitivity, is a problem that still exists.

Heterologous enzyme immunoassay systems with minor structural differences between the hapten carrier conjugate and the enzyme conjugate that were used as immunogen and tracer, respectively, presents a good alternative to homologous assays in achieving the target sensitivity. The influence of such heterology on estrogen enzyme immunoassay has been studied.[15] The assay specificity is a virtue that is largely affected by the site of attachment, but more specific assays have been developed using different bridge length linkers.[16–18] In the present work, we have also used antigen heterology for the first time to develop a highly specific ELISA for the measurement of E1-3-G in urine samples. Furthermore, urea has been used as a linker in the tracer to evaluate its effects on the assay parameters.

EXPERIMENTAL

Chemicals, Reagents and Instrumentation

All solvents, chemicals, and salts used in the present study were of analytical grade. All the steroids used for cross-reactivity were from Sterloids, Inc., P. O. Box 689, Newport, RI 02840-0600, USA. Bovine serum albumin (BSA), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), Freund's complete adjuvant (FCA), gelatin, and thimerosal were purchased from Sigma Chemical Company, St. Louis, MO, USA; Horse radish peroxidase (HRP) and tetramethylbenzidine/H_2O_2 solution were purchased from Bangalore Genei, Bangalore, India and Arista Biochemical, USA, respectively. Microtitre plates were procured from Greiner, Germany.

Buffers

a. The most frequently used buffer was 10 mM phosphate (10 mM PB), pH 7.0, ($Na_2HPO_42H_2O$: 0.895 gm/L and $NaH_2PO_42H_2O$: 0.39 gm/L) containing 0.9% NaCl (10 mM PBS) and 0.1% NaN_3.
b. HRP conjugate dilution buffer was 10 mM acetate buffer (10 mM AB), pH 5.6 (CH_3COONa: 0.84 gm/L and 1 N CH_3COOH 1.5 mL/L), containing 0.1% thimerosal and dextran T-70, 0.3% BSA
c. Microtitre well blocking and stabilizing buffer was 10 mM PB containing 0.9% NaCl, 0.2% BSA, 0.1% gelatin, thimerosal, dextran T-70, ethylene diamine tetra acetic acid di-potassium salt (EDTA: K salt), and 0.01% gentamicin sulfate.

Methods

Sample Collection, Storage and Dilution

Daily specimens of early morning urine (the first voiding upon rising) were collected from a healthy non-pregnant female volunteer throughout her complete menstrual cycle and stored at $-20^{\circ}C$. The samples were diluted 20-fold in enzyme conjugate buffer for running ELISA.

Preparation of E1-3-G-BSA Conjugates

Estrone-3-glucuronide was coupled to BSA by an active ester method with modification.[19] To 5 mg of E1-3-G, 200 μL of each, dioxan and dimethyl

formamide was added. To this solution, 100 μL of water containing 10 mg NHS and 20 mg EDAC was added; the reaction mixture was kept for activation at 4°C for 24 hrs. Activated E1-3-G solution was added to the aqueous solution of 100 mg of BSA (1 mg/0.3 mL), vortex-mixed, and kept for 24 hrs at 4°C. The E1-3-G-BSA conjugate was dialyzed against 3–4 changes of water. The dialysate was frozen, lyophilized, and kept at 4°C in aliquots of (1 mg) for immunization.

Preparation of Nandrolone-17-HS-Peroxidase Conjugate

The nandrolone-17-hemisuccinate (N-17-HS) was directly conjugated to HRP by an activated ester method with modification.[19] To 5 mg of N-17-HS, 200 μL of each, dioxan and dimethyl formamide, was added. To this solution, 100 μL of water containing 10 mg NHS and 20 mg EDAC was added; the reaction mixture was kept for activation at 4°C for 24 hrs. Activated N-17-HS solution was added to the aqueous solution of HRP (1 mg/mL) and kept for 24 hrs at 4°C. After incubation, the reaction mixture was passed through a G-25 column, which was previously equilibrated with 10 mM PBS containing 0.1% thimerosal. The brown colored fractions containing enzyme activity were pooled and, to it, 1% of sucrose, ammonium sulfate, BSA, and an equal volume of ethylene glycol were added. The solution was kept at −30°C in aliquots for future use.

Preparation of Nandrolone-17-HS-Urea-HRP Conjugate

a. Conjugation of urea to HRP: HRP (2 mg), NHS (4 mg) and EDAC (8 mg) were dissolved in 200 μL of water. This reaction mixture of HRP was kept at 4°C overnight. To the activated HRP solution, 20 mg of urea was added and further incubated at 4°C for overnight. The HRP-urea solution was dialyzed against two changes of water.
b. Conjugation of nandrolone-17- hemisuccinate to urea-HRP: To 5 mg of N-17-HS, 200 μL of each, dioxan, and dimethyl formamide was added. To this solution, 100 μL of water containing 10 mg NHS and 20 mg EDAC was added; the reaction mixture was kept for activation at 4°C for 24 hrs. Activated N-17-HS was then added to HRP-urea and allowed to react at 4°C for 24 hrs. After incubation, the reaction mixture was passed through a G-25 column which was previously equilibrated with 10 mM PBS containing 0.1% thimerosal. The brown colored fractions containing enzyme activity were pooled and, to it, 1% of sucrose, ammonium sulfate, BSA, and an equal volume of ethylene glycol were added. The solution was kept at −30°C in aliquots for future use.

Immunization

E1-3-G-BSA (1 mg) was dissolved in saline (0.5 mL) and emulsified with Freund's complete adjuvant (0.5 mL). Subcutaneous injections were given to the New Zealand white rabbits at multiple sites. The weekly injections, for 5 weeks, were followed by monthly booster injections. The rabbits were bled 10 days after the booster injection. Antiserum was collected after centrifugation at 2,500 rpm for 10 minutes and was stored at -30°C.

Coating of Antibody to Microtitre Plates

The 96-well microtitre plate was coated using the immunobridge technique for primary antibody immobilization described elsewhere.[20] In brief, 250 μL of the normal rabbit serum (NRS) diluted (1:250) in water was dispensed into each well and incubated at 37°C overnight. Following incubation, the plate was washed under running tap water. To the NRS coated wells, 250 μL of 1:1,000 diluted goat anti rabbit gamma globulin (ARGG) was added and incubated for 2 hrs at 37°C. After incubation, the contents of the plate were decanted and washed under running tap water. To the ARGG coated microtitre plates; 200 μL of appropriately diluted (1:4,000) E1-3-G antiserum in buffer "A" was dispensed. The plate was left at 37°C for 2 hrs. Unadsorbed antibody was then washed off and 250 μL of buffer "C" was then added to block the unoccupied sites of the plate. The plate was kept at 37°C for 1 h. The contents were decanted and the plate was dried at RT and kept at 4°C for future use.

Assay Procedure

To the antibody-coated wells, 50 μL of 1:20 diluted urine samples; E1-3-G standards (0–50 ng/mL) were added along with 100 μL of N-17-HS-HRP/N-17-HS-Urea-HRP enzyme conjugate in duplicate. The plate was incubated at room temperature for 1 hr. Unbound contents were decanted and the plate was washed in running tap water by filling, decanting, and flicking. To measure bound enzyme activity, 100 μL of TMB/H_2O_2 substrate solution was added to all wells and incubated for 15 minutes. The reaction was stopped by adding 100 μL 0.5M H_2SO_4 and the color intensity was measured at 450 nm in a Tecan-Spectra ELISA plate reader.

RESULTS

Sensitivity

The lower detection limit of the assay, i.e., concentration equivalent to A_0-2SD, was calculated after 20-fold determination of A_0 binding (binding at

zero dose). The dose-response curves of the N-17-HS- HRP and N-17-HS-urea-HRP with E1-3-G antibody are illustrated in Fig. 1. The sensitivity of the assay with N-17-HS-HRP and N-17-HS-Urea-HRP was 0.6 ng/mL and 0.4 ng/mL, respectively.

Notwithstanding is the decrease in the effective displacement doses, i.e., ED_{50} from 20 ng/mL to 2 ng/mL, with introduction of urea linker to the enzyme conjugate.

Specificity

The specificity of the E1-3-G-BSA antibody was estimated as the percentage of cross-reaction with commercially available steroids of similar molecular structures. The % cross reaction of E1-3-G-BSA antibody was less than 0.1% with naturally occurring C_{18}, C_{19}, C_{21}, and C_{27} steroids with N-17-HS-HRP enzyme conjugate. The cross-reaction of E1-3-G antibody using N-17-HS-Urea-HRP against all the analogous steroids also remained below 0.1%. There is a unique feature in the assay, that is, no cross-reaction was observed when N-17-HS was itself tested as a cross reactant.

Recovery and Precision

The recovery and precision of the assay were determined using N-17-HS-Urea-HRP as enzyme conjugate, keeping in view the high sensitivity and lesser ED_{50}.

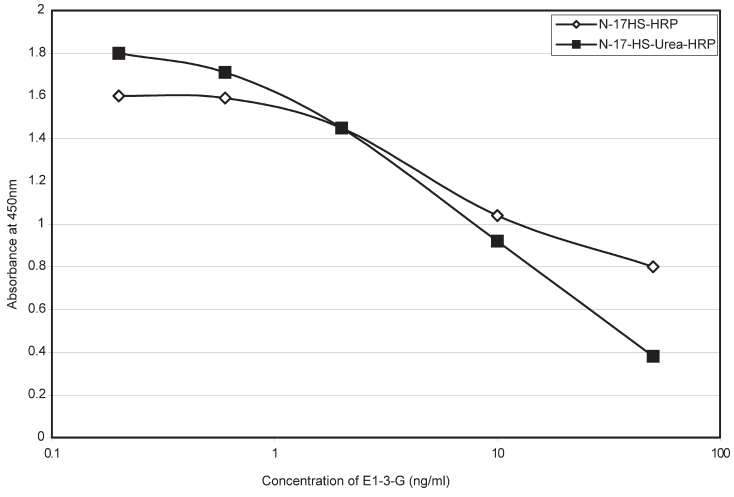

Figure 1. Dose-response curves of E1-3-G using E1-3-G -BSA antibody with N-17-HS-HRP and N-17-HS-Urea-HRP enzyme conjugates.

Recovery

The recovery was calculated as the percentage of the added mass recovered from a 1:20 diluted male urine sample. The varying amounts of E1-3-G (2.5–15 ng/mL) were spiked into the urine samples to assess the accuracy. The recovery of the assay was in the range of 96–103%, as depicted in Table 1.

Precision

The level of imprecision is estimated by examining the intra-assay and inter-assay variation. Three urine pools of low, medium, and high E-1-3G concentrations were run in five consecutive assays. The intra- and inter-assay coefficients of variation ranged from 4.7–9.0% and 5.1–6.4%, respectively. Table 2 gives the precision profiles for E1-3-G assay.

Excretion Profile of E1-3-G during the Ovulatory Cycle

The pattern of E1-3-G concentration was analyzed in the early morning urine samples collected during the four consecutive cycles of a normally mensturing woman. The excretion profile is depicted in Fig. 2. A clearly demarcated peak reflects the E1-3-G upsurge during the ovulatory phase of the menstrual cycle.

Correlation Coefficient

The E1-3-G was also measured in the urine samples that were heat denatured at 60°C for 30 min after 20-fold dilution. A very negligible difference was observed in the concentration of E1-3-G between the non-denatured and heat denatured cycle. The correlation coefficient between the heat denatured and non-denatured sample was 0.94, i.e., $r = 0.94$ ($n = 27$).

Table 1. Recovery of added E1-3-G from 1:20 diluted urine samples

Urine pool no.	E1-3-G added (ng/mL)	E1-3-G expected (ng/mL)	E1-3-G observed (ng/mL)	Recovery (%)
Pool A	—	—	2.71	—
Pool B	2.5	5.21	5.41	103
Pool C	5.0	7.21	7.44	96.4
Pool D	15.0	17.21	17.37	101.5

Table 2. Inter and intra assay coefficient of variation for the measurement of E1-3-G in three pools

Variation	Sample value	Coefficient of variation (%)
Intra-assay	4.24 ± 0.2	4.7
N = 8	7.11 ± 0.38	5.3
	18.12 ± 1.63	9.0
Inter-assay 5*	4.5 ± 0.23	5.1
	7.4 ± 0.39	5.2
	18.75 ± 1.2	6.4

N = Number of times same sample analyzed for intra-assay variation.
* = Number of times assays carried out for inter-assay variation.

DISCUSSION

In the present paper, we have described the ELISA for the measurement of E1-3-G, an important index of predicting the duration of the fertile period in urine samples. The assay uses less volume of standards/samples (50 μL) and enzyme conjugate (100 μL) and takes 2 hrs for its completion, which includes the processing of samples too. The distinctive feature of the assay is the use of the nandrolone-17-hemisuccinate derivative for labeling with the enzyme. This molecule doesn't show any cross reaction with the antibody in unlabeled form but, once it is labeled with the enzyme, a very good displacement curve

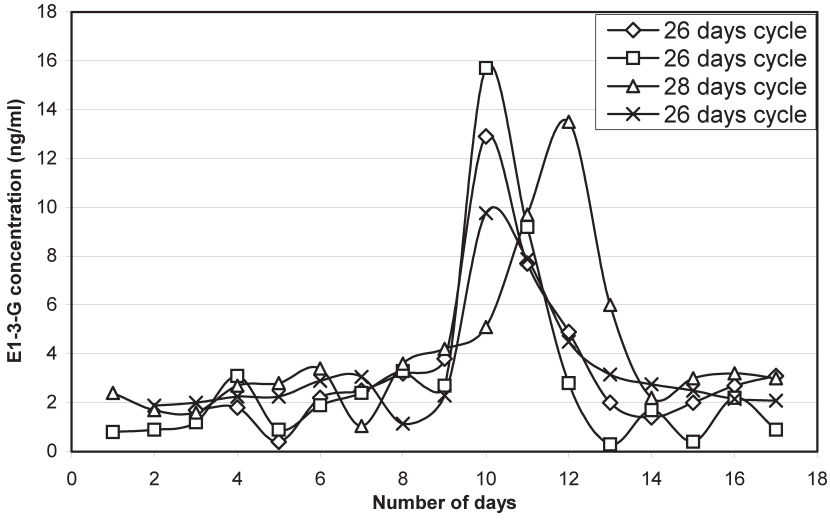

Figure 2. Pattern of E1-3-G concentration in female early morning urine.

is obtained. Hence, this hapten heterologous combination of immunogen and enzyme conjugate is quite unique in this respect, from various studies that have been done on heterologous systems using different steroids.

Van Weeman and schuurs[21] reported a high degree of cross-reaction in heterologous systems due to the presence of various subpopulations of antibodies with higher affinity for heterologous hapten. However, we have observed a low cross-reaction of E1-3-G-BSA antibody against the analogous steroids in our assay system. This may be attributed to the different electrostatic interactions between the binder and the competing analytes.

The antibody raised against E1-3-G-BSA has differential affinity for the enzyme conjugate and the analyte. Thereby, a low amount of unlabeled analyte was able to displace the enzyme labeled hapten (N-17-HS-Urea-HRP) and a lower detection limit of 0.4 ng/mL and ED_{50} of 2 ng/mL was achieved. Piran et al.[22] demonstrated that the effect of hapten heterology on assay sensitivity is probably due to the increased steric accessibility of the free analyte to antibody and also suggested that cooperative binding of analyte with the antibody could also be the reason for improved sensitivity.

Various studies have been done earlier to emphasize on the role played by introducing bridges in the enzyme conjugates.[16–18] In our experiments, we have incorporated urea as a homo-bifunctional bridge in the enzyme conjugate. The results were encouraging with a decrease in the effective displacement dosage (ED_{50}) from 20 ng/mL to 2 ng/mL, making the slope of the assay steeper. The lower detection limit was also improved to 0.4 ng/mL. Therefore, analytical validation of the assay was performed using N-17-HS-urea-HRP enzyme conjugate. This immunoassay also satisfied the other criteria for reliability, viz., recovery and precision, where intra- and inter-assay coefficients of variation lie in the range of 4.7–9.0% and 5.1–6.4%, respectively. The recovery of the assay was in the range of 96–103%.

The estrone glucuronide concentration, as measured by present assay in diluted urine samples of four ovulatory cycles of a female volunteer, correlated well, as reported by others. The monthly cycles showed a marked preovulatory surge in E1-3-G levels on 10/12th day. The urine samples were denatured at 60°C and correlated with non-denatured samples of the same cycle to further validate the assay. The correlation coefficient (r) was 0.94.

In conclusion, heterologous combinations could be looked upon as an effective approach for developing assays having good specificity, along with significant sensitivity.

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