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USE OF A MONOCLONAL ANTIBODY TO ESTRONE-3-GLUCURONIDE IN AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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Summary—A direct urinary ELISA for estrone-3-glucuronide has been produced following cloning and characterisation of a monoclonal antibody to the above estrogen metabolite. The ELISA follows our established pattern of absorbing a thyroglobulin conjugate, to which estrone-3-glucuronide has been coupled, to the wells of a microtitre plate using guanidine hydrochloride. A competition reaction between either standards/samples and the adsorbed hormone compete for antibody combining sites. The assay is completed by addition of an anti-mouse Ig-peroxidase complex and read at 492 nm following additions of *O*-phenylenediamine substrate in under 4 h. The correlation between urinary "total estradiol" and "total estrone and estradiol" is very good and, in conjunction with our ELISA for pregnanediol glucuronide, has allowed for the improved clinical management of infertile and subfertile women.

INTRODUCTION

The measurement of estrogen and progesterone metabolites in urine has had a renaissance within the last five years for a variety of reasons. The World Health Organization (W.H.O.) has embarked on a study [1, 2] with others [3] of estrogen metabolites in order to provide better understanding of, and possible improvement in, natural family planning methods. *In vitro* fertilization techniques have also been vigorously pursued in recent years. As a consequence, the detection of ovulation and the monitoring of ovarian activity has given a boost to research into urine hormone metabolite concentrations [4–7] which reflect plasma hormone levels without the inconvenience of daily venepuncture. Over this time period, we have successfully introduced enzyme-linked immunosorbent assay technology (ELISA) to our laboratory [8, 9] and have recently produced monoclonal antibodies which have led to significant improvements in time and cost efficiencies [10]. Here we report the production and characterization of a monoclonal antibody to estrone-3-glucuronide, and the development of a direct urinary ELISA coupled with an ELISA for pregnanediol-3-glucuronide [11], which has helped our clinicians to address some problems associated with infertility.

EXPERIMENTAL

Reagents

All steroids used for synthesis and cross-reactivity purposes were purchased from Sigma Chemical Co. (St Louis, Mo.), while all other chemicals were AR grade. The immunogen estrone-3-glucuronide-BSA was prepared by coupling estrone-3-glucuronide to BSA via the mixed anhydride method [12], and the same method was used to prepare the estrone-3-glucuronide-thyroglobulin conjugate [8]. Goat-anti-mouse IgG-peroxidase conjugate was purchased from Tago Inc. (Burlinghame, Calif.), while the mouse monoclonal isotyping kit was from Amersham (Bucks, England). Falcon 3912 Microtest III 96-well microtitre plates and gelatin, bacteriological grade were from Becton Dickinson Co. (Oxnard, Calif.). Reagents used in cell fusion and culture were all purchased from Sigma (St Louis, Mo.).

Buffers

Phosphate-buffered saline (PBS) 0.05 mol/l NaH_2PO_4, 0.15 mol/l NaCl adjusted to pH 7.4 with 5 mol/l NaOH containing Tween-20 (1 ml/l) was used for washing the microtitre plates. Assay buffer consists of wash buffer containing Tween-20 (1 ml/l) and gelatin (1 g/l) (PBS/Gel) and is used in all other stages of the assay. Thiomersal was added to the PBS/Gel as a preservative (1 g/l).

Substrate buffer consisted of 50 mmol/l Na_2HPO_4, 25 mmol/l citric acid, pH 5.0. Enzyme substrate solution was prepared freshly by dissolving 40 mg of *O*-phenylenediamine in 100 ml of substrate buffer and immediately prior to use 60 μl H_2O_2 was added.

Immunization and cell culture

Female RBF/DN mice purchased from The Jackson Laboratory (Bar Harbor, Minn.) were injected i.p. with 50–100 μg of estrone-3-glucuronide-BSA/2 ml of Freund's complete adjuvant for a period

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of three months at 4-weekly intervals. Following asceptic removal of the spleen, lymphocytes were fused with FOX-NY [13] myeloma cells at a ratio of 5:1 respectively, using Taggart hybridoma technology from Hyclone Laboratories (Logan, Ut.). The cells were resuspended in RPMI medium containing 10% fetal calf serum (v/v) 2 mM L-glutamine and AAT (adenine:aminopterin:thymidine, $75:0.08:16 \times 10^{-6}$ M respectively) after fusion, and placed in 96-well Nunc culture plates at a concentration of 10^5 cells/well. Positive hybrids were cloned twice by limiting dilution and grown in 50 ml culture flasks to confluence, at which time the supernatants were harvested and centrifuged prior to use. Immunoglobulin class and subclass were determined by the isotyping kit.

Estrone-3-glucuronide ELISA and hybrid screening procedure

The hybrid myeloma screening procedure is essentially the same as for the ELISA assay except that supernatant from the microtitre plate is substituted for patient samples in the ELISA assay. All other steps are the same.

Microtitre plates are coated overnight with 100 μl of aqueous 6 M guanidine hydrochloride/well containing estrone-3-glucuronide thyroglobulin (250 pg/ml) at 4°C. The plates are washed four times with wash buffer (PBS) and blocked for 1 h with 150 μl/well of PBS/Gel. Following aspiration of "block", 50 μl of assay standard or sample are added to the appropriate well. Antibody supernatant 6C diluted 1:150 in assay buffer is added to each well (50 μl) and left to incubate for 2 h at ambient temperature. The wells are further washed 4 times with PBS wash buffer and 100 μl of goat-antimouse IgG-HRP in assay buffer (1/1000) added to each well and left to incubate for 1.5 h. Finally, the plates are washed 4 times again and substrate added. Color development is stopped by the addition of 1.25 M H_2SO_4/well and the absorbance read at 492 nm. All the above steps are carried out automatically on a Behring ELISA Processor II (Hoechst, Marburg, F.R.G.) with the exception of addition of standard and patient samples. Patient samples are prepared for ELISA determinations in the following way.

Early morning urine (EMU) samples [14] were diluted 1:50 in assay buffer and a 50 μl aliquot dispensed into each microtitre plate well. Samples from women receiving infertility treatment occasionally required further dilutions for results to be interpolated from the standard curve. Standards were serially diluted in assay buffer from a stock 1 mg/ml solution of estrone-3-glucuronide to give seven standards (0–500 pg/50 μl).

Recovery experiments were carried out by adding known amounts of estrone-3-glucuronide to male urine in the range 0–400 nmol/l and subsequently assayed. Prior to the setting up of the assay, estrogens in urine were analysed in this laboratory by the Bio-Merieux kit which enzymatically determines total estrone and estradiol in both plasma and urine after hydrolysis of glucuronides and sulphates [15].

Cross-reactivity studies were carried out according to the method of Abrahams[16]. Ten normal women were followed for one complete menstrual cycle, and women patients undergoing a variety of fertility protocols were assayed, and results compared with those derived from the kit method.

RESULTS

Two high affinity clones secreting estrone-3-glucuronide antibodies from the original fusion plates were studied further. Both clones 7E and 6C gave very good curves, but 6C had a superior cross-reactivity profile (Table 1). Figure 1 shows the standard curve generated from Mab 6C. The sensitivity was calculated by taking two standard deviations from the zero binding point optical density and interpolating from the standard curve, giving a result of less than 1 nmol/l. Spiking recoveries shown in Table 2 are the mean of eight sets of different duplicates for each value from two separate experiments. Intra- and inter-assay CVs for 10 sets of duplicates are shown in Table 3. Figure 2 shows data from a hysterectomized female with a 31 day ovulatory cycle. The estradiol results are total estradiol after hydrolysis of glucuronide and sulphate moieties. Data from three normal women are shown in Fig. 3 together with pregnanediol-3-glucuronide results. All EMU results were adjusted for concentration relative to creatinine assuming 10 mmol creatinine excretion over 24 h [17]. Figure 4 shows a comparison between estrone-3-glucuronide and total estradiol and estrone results for female patients who

Table 1. Cross-reactivities of supernatants C6 and E7 by ELISA

Steroid	Supernatant C6	E7
Estrone-3-glucuronide	100	100
Estradiol-3-glucuronide	0.5	1.0
Estriol-3-glucuronide	<0.1	<0.1
Estrone-3-sulphate	<0.1	0.1
Estradiol-3-sulphate	<0.1	<0.1
Estriol-3-sulphate	<0.1	<0.1
Estrone	0.3	1.0
17β-Estradiol	<0.1	0.1
Estriol	<0.1	<0.1
Pregnanediol-3-glucuronide	<0.1	<0.1
5β-Pregnene-3α-11β-17α-triol-20-one	<0.1	<0.1
4-Pregnene-11β-17α-diol-3,20-dione	<0.1	<0.1
4-Pregnene-20α-ol-3-one	<0.1	<0.1
Prenene-3β-ol-20-one	<0.1	<0.1
Testosterone	<0.1	<0.1
Progesterone	<0.1	<0.1
Cortisol	<0.1	<0.1
11-Dehydrocorticosterone	<0.1	<0.1
17α-OH-Progesterone	<0.1	<0.1
11-Deoxycortisol	<0.1	<0.1
Deoxycorticosterone	<0.1	<0.1
Corticosterone	<0.1	<0.1
21-Deoxycortisol	<0.1	<0.1
Cortisone	<0.1	<0.1
Androstenedione	<0.1	0.7
Androsterone	<0.1	1.4
Isotype	IgG2a K	IgG2a K

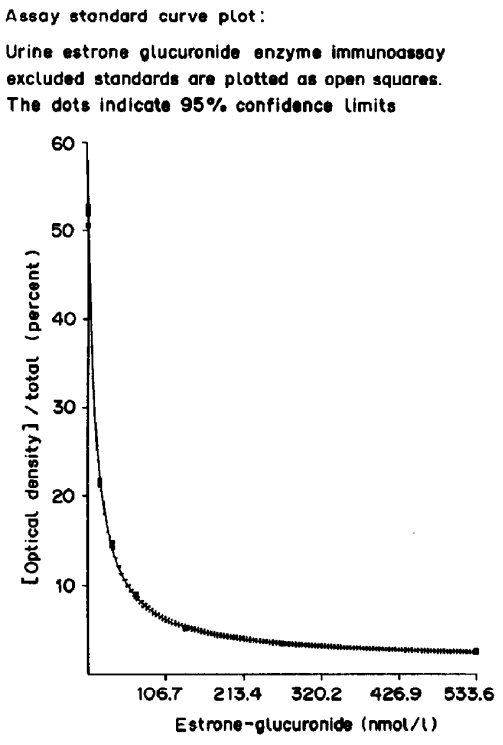

Fig. 1. Standard curve for urine estrone-3-glucuronide ELISA. 95% confidence limits are indicated by dotted lines.

are under investigation for infertility. In all cases, the results given are the mean of duplicate derminations.

DISCUSSION

The assay of estrogen and progesterone metabolites to monitor ovarian activity has been changing in the last few years from radioimmunoassays (RIA) [18–20] to enzyme immunoassays (EIA) [21] and ELISAs [14, 15, 17], as the sensitivity problem which beset early ELISA assays was overcome .and the move away from radionucleides, for both environmental and economic reasons, has been largely completed. To date, most immunoassay techniques that do not rely on radioisotopes, have the synthesis of a hormone–enzyme conjugate as a necessary prerequisite. The synthesis of these conjugates is not always easily accomplished in a routine hospital laboratory and this step has to be continually repeated as the enzyme activity decreases over a period of months. This loss of enzyme activity is analogous to low specific activity in RIA. Here we present the development of a direct ELISA on EMU diluted urine specimens with a monoclonal antibody of high specificity and requiring only the synthesis of a hormone-thyroglobulin conjugate. This method confers a number of advantages over the usual ELISA assay setup, which include stability of conjugate (>3–4 yr) and use of a common second antibody–enzyme conjugate which can be purchased cheaply from commercial companies.

Table 2. Spiking analysis of male urine. Each value is the mean of 8 sets of duplicates

Amount added (nmol/l)	Amount recovered (nmol/l)	% Recovery
400	456	111
200	210	98.5
100	128	114
50	74	120
25	42	114
0	14	—

Table 3. Intra- and inter-assay variation of urine pools. Duplicate determinations

	Pool			
Inter-assay variation	1 (n = 11)	2 (n = 10)	3 (n = 10)	
Mean ± SD (nmol/l)	14.6 ± 1.1	44.9 ± 2.7	69.4 ± 5.4	
CV%	7.1%	5.9%	7.7%	
	Pool			
Inter-assay variation	1 (n = 10)	2 (n = 10)	3 (n = 10)	4 (n = 10)
Mean ± SD (nmol/l)	26.6 ± 1	44.0 ± 2.0	70.1 ± 2.4	83.3 ± 4.6
CV%	3.7%	4.5%	3.4%	5.5%

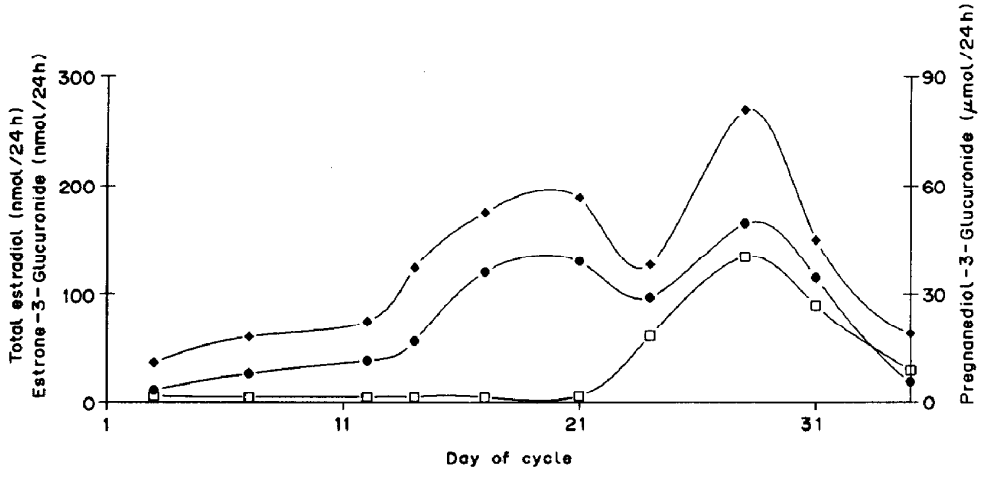

Fig. 2. Urinary excretion pattern for estradial ◆—◆, estrone ●—● and pregnanediol □—□ as determined by kit and ELISA methods.

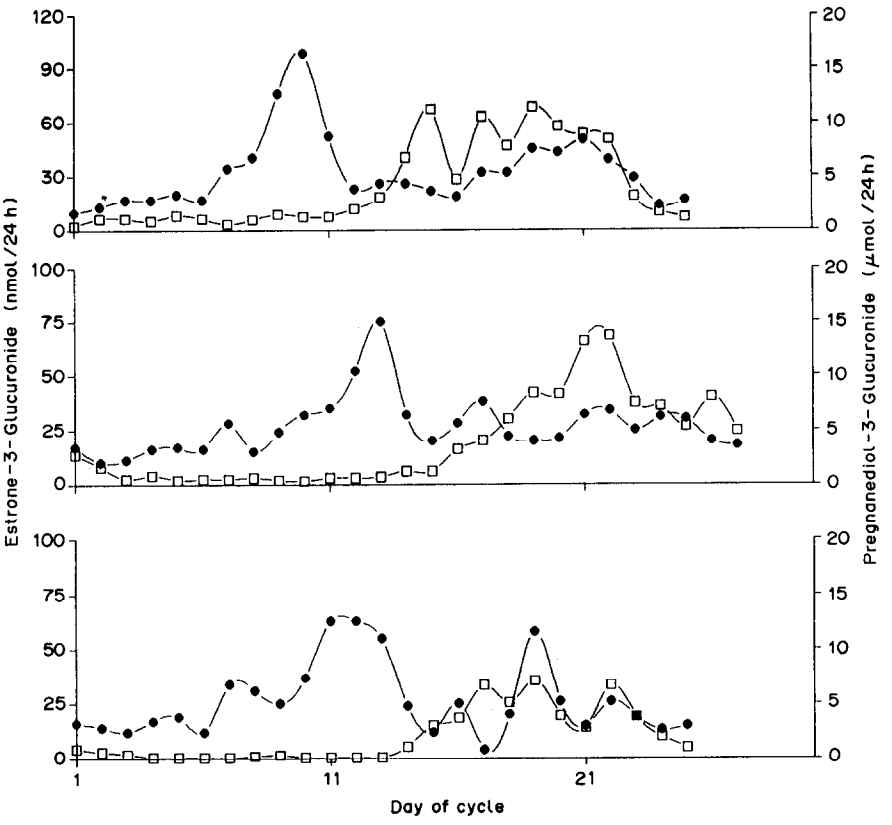

Fig. 3. Urinary excretion pattern of estrone-3-glucuronide ●—● and pregnanediol-3-glucuronide □—□ for 3 normal cycling women.

Data by other groups [17] has established that measurement of estrogen metabolites in EMU accurately reflects total urine estrogen excretion and parallels the parent hormone plasma profile. In conjunction with an ELISA for pregnanediol-3-glucuronide [11], we have established a system which allows our clinicians to monitor ovarian activity and infertility problems, easily, cheaply and with a high degree of confidence—a system which has obvious commercial potential [22].

Acknowledgement—We thank Mrs S. Harrison Sands for secretarial assistance.

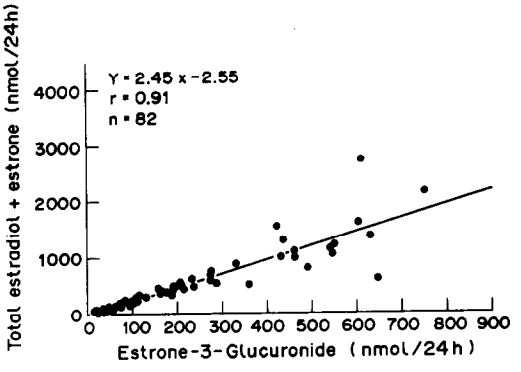

Fig. 4. Correlation of urine estrone-3-glucuronide concentrations by ELISA versus estradiol + estrone concentrations by kit method.

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