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The Measurement of Oestrone-3-glucuronide in Urine by Non-Competitive Idiometric Assay

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We report a novel non-competitive idiometric assay for the measurement of oestrone-3-glucuronide (EG) in diluted urine. The method is based on the use of two types of anti-idiotypic antibody, the β-type and α-type, that recognize different epitopes within the hypervariable region of the primary anti-EG antibody (Ab_1). The β-type anti-idiotypic antibody is analyte sensitive and competes with the analyte for an epitope of the primary antibody at the binding site. On the other hand, the α-type is analyte insensitive, but does not bind the Ab_1 in the presence of the β-type due to epitope proximity. In the present format, reaction mixtures containing the europium labelled Ab_1 are reacted sequentially with EG standards or diluted urine samples, with the β-type anti-idiotypic antibody and biotinylated α-type anti-idiotypic antibody on immobilized streptavidin coated microtiter plates. After 1 h incubation, the fluorescence of europium is measured by a time-resolved fluorescence and is proportional to the concentration of EG over a range of 0–10 nmol/l. The method demonstrates good sensitivity, precision and comparability with an alternative competitive fluorescent immunoassay. The idiometric assay for EG may be applied for the monitoring of ovarian function in women and is suitable for dipstick technology.

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INTRODUCTION

It has been suggested by several investigators [1–3] that measurement of urinary metabolite of oestradiol, oestrone-3-glucuronide (EG) may be used to monitor ovarian function in the human female. Accordingly, several methods based on competitive immunoassay procedures, using a variety of labels [1, 4, 5]) have been developed for the measurements of EG in diluted urine and the usefulness of the measurement of urinary EG to predict ovulation and to delineate the fertile period has been explored [6, 7]. The major limitation to most of the competitive-type immunoassays is that the response is inversely proportional to the concentration of the analyte, and the assay has limited sensitivity [8–10]. As a consequence, these methods are less amenable for dipstick technology and home use.

We [11, 12] and others [13, 14] have recently reported the development of a non-competitive immunoassay for the measurement of small molecules which we termed idiometric assay. The development of the idiometric assay, as exemplified by the measurement of oestradiol in serum, was achieved by the identification and utilization of two types of anti-idiotypic antibodies that recognize different epitopes within the hypervariable region of the specific primary anti-oestradiol antibody (Ab_1). The first anti-idiotype, the β-type, is analyte sensitive while the second anti-idiotype, the α-type, is analyte insensitive (see Fig. 1). Moreover, the α-type that has been selected for the idiometric assay will not bind Ab_1 in the presence of the β-type because of steric hindrance. The identification of a matching pair of β-typic and α-typic anti-idiotypic antibodies was achieved by two-site epitope analysis of antigenic epitopes of the primary Ab_1, similar to that used in epitope-mapping experiments [15]. The idiometric assay for oestradiol was shown to be an excess-reagent assay and had a wide working range [11, 12]. We now report a variant of the idiometric assay that is amenable to measurement of EG by dipstick technology. In the present format, the europium labelled anti-EG antibody (Ab_1) is reacted sequentially with samples or standards, followed with the β-type which blocks the unoccupied sites, and then with the biotinylated α-type in streptavidin

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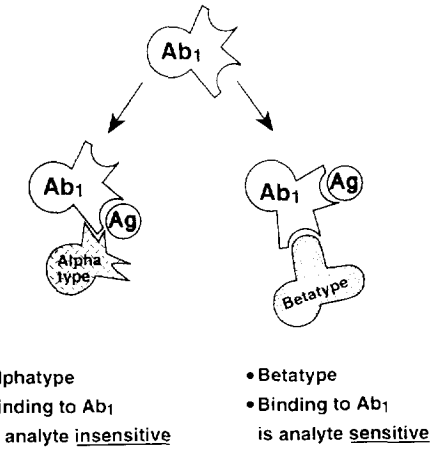

Fig. 1. Schematic representation of the various anti-idiotypic antibodies induced by Ab_1.

immobilized microtiter plates. After a 1 h incubation and a wash step, the plates are processed for time-resolved fluorescence (see Fig. 2). The response, i.e. the fluorescence that is measured, is proportional to the dose of EG over a range of 0–10 nmol/l.

MATERIALS AND METHODS

Reagents

Steroids, Freund's adjuvant, mouse serum, pristane, biotin-ε-aminocaproyl-γ-butyryl-*N*-hydroxysuccinimide ester and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were purchased from Sigma Chemical Co. (St Louis, MO). Sepharose-Protein A and Sephadex G-25 were purchased from Pharmacia (Uppsala, Sweden). Keyhole Limpet Hemocyanin (KLH) was obtained from CalBiochem (La Jolla, CA). The labelling reagent, *N*-1(*p*-isothiocyanatophenyl)-diethylenetriamine-N^1, N^2, N^3-tetraacetate (DTTA) chelated with Eu++, Streptavidin coated microtiter plates and enhancement solution were kindly provided by Dr Ilkka Hemmilä and Dr Kim Petterson (Wallac Oy, Turku, Finland).

Assay buffer and wash solution were prepared as described previously [11].

Preparatory methods

Rabbit anti-mouse IgG-coated microtiter plates. Rabbit anti-mouse IgG was adsorbed at acidic pH onto the walls of microtiter plates as described previously [11, 16].

Europium-labelled antibodies. Anti-EG (rat-mouse hybridoma, IgG_1 class, clone # $3F_{11}$) [17] was propagated as ascites in irradiated CD_2 male mice which were primed with pristane. Ascites containing monoclonal antibodies to EG were purified by chromatography on Sepharose-Protein A. Briefly, 1 ml of ascitic fluid was diluted with 1 ml of 100 mmol/l sodium phosphate buffer (pH 8.0) and applied to the column. The IgG fraction was eluted from the matrix with 100 mmol/l sodium acetate solution (pH 6.0). The IgG fraction was then dialysed against 10 mmol/l sodium phosphate buffer (pH 7.4) containing 150 mmol of sodium chloride. The concentration of the IgG fraction was then determined, taking 1.4 O.D. at 280 nm as 1 mg protein.

One part of the IgG fraction (1 mg) was labelled with europium chelate as described previously [11, 16], and the rest was stored at −20°C until use. For screening purposes, another irrelevant rat–mouse hybridoma (clone $4A_9$) of the same isotype as clone $3F_{11}$, was also labelled with europium.

Preparation of monoclonal anti-idiotypic antibodies against anti-EG. The immunogen was prepared by coupling anti-EG to KLH. For this purpose, purified anti-EG IgG (5 mg) in 1 ml phosphate buffered saline (PBS), pH 7.4, is mixed with a 10,000:1 molar ratio of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) to IgG. The mixture is stirred at 4°C for 30 s. KLH is added at a molar ratio of 50:1 IgG to the IgG–EDAC reaction mixture which is stirred for 2 h at 25°C and then overnight at 4°C. The mixture is then dialysed against PBS overnight at 4°C and stored at −20°C until use.

For immunization purposes female CD_2 mice (age: 2 months) were immunized with anti-EG–KLH conjugate (50 μg/mouse) in complete Freund's adjuvant. Subsequently, one booster injection was given using the IgG–KLH conjugate in incomplete Freund's adjuvant. After 10 days of the booster injection, the anti-idiotypic response was checked as described previously [11]. Two and half months after the initial immunization the spleen cells of the CD_2 mouse showing the highest serum titer of antibodies which recognized europium labelled anti-EG IgG were fused with a mouse myeloma cell line NSO, kindly donated by Dr Milstein, (Cambridge, U.K.), using the hybridoma technique of Köhler and Milstein [18]. The culture supernatants of growing hybridomas were screened for antibody activity using the following screening procedures.

Screening experiments

Hybridomas secreting xenotype, β-type and allotype anti-idiotypic antibodies were identified using four screening strategies, as follows.

Screening assay 1: identification of IgG secreting hybridomas. In this screening assay, hybridoma culture supernatants (50 μl/well) were added to the anti-mouse IgG coated microstrips containing 150 μl of assay buffer/well. The plates were incubated for 1 h and subsequently washed three times. The plates were then blocked with 10 μl of mouse serum and subsequently 200 μl of europium-labelled anti-EG IgG (clone $3F_{11}$, approx. 10^7 cps/ml) were added to each well. The plates were incubated for 1 h and subsequently processed for time resolved fluorescence

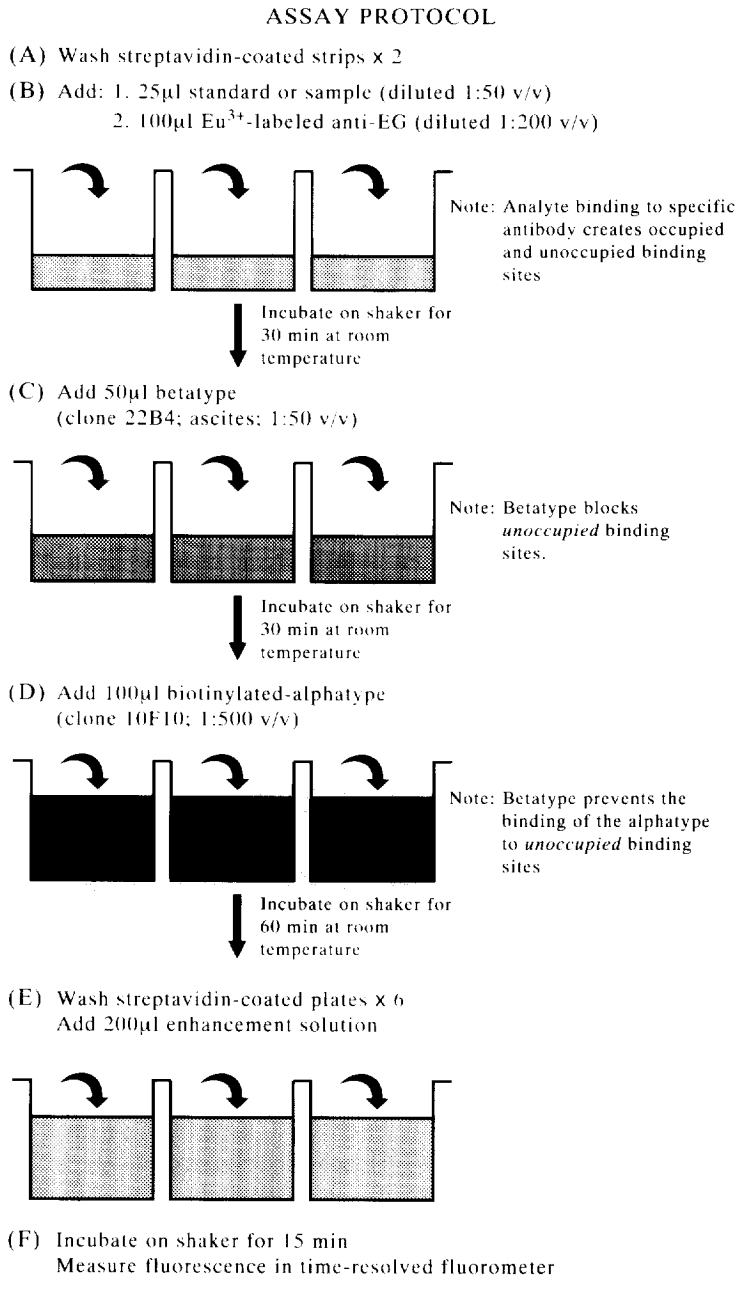

Fig. 2. The assay protocol of the idiometric assay.

[11]. The wells that gave positive results were classified as anti-xenotypic and anti-idiotypes.

Screening assay 2: identification of xenotype secreting hybridomas. In the second screening procedure, the hybridomas that gave positive results in the first screening assay were added (50 µl/well) in duplicate to anti-mouse IgG coated strips containing 150 µl of assay buffer. The plates were incubated, washed and blocked as described in the first screening procedure (see above). Subsequently, 200 µl of assay buffer containing europium-labelled clone $4A_9$ (10^7 cps/well; same isotype as anti-EG clone $3F_{11}$), were added to each well. The plates were processed as described in the first screening procedure. Immobilized hybridoma culture supernatants that failed to bind europium-labelled clone # $4A_9$ were classified as anti-idiotypes whereas those that gave positive results were classified as xenotypic antibodies.

Screening assay 3: identification of β-type and α-type secreting hybridomas. In this screening procedure, the 23 hybridomas, classified as anti-idiotypes, that gave negative results in the second screening assay were added (50 µl/well) in quadruplet to anti-mouse IgG coated microtiter wells containing 150 µl of assay buffer. The plates were incubated for 2 h, washed and blocked as described in the first screening procedure. Subsequently, to one set of duplicates, 200 µl of assay buffer containing europium-labelled anti-EG, clone $3F_{11}$, (10^7 cps/ml) were added and to the other set of duplicates, 200 µl of assay buffer containing an excess

of EG (100 ng/well) and europium-labelled anti-EG, clone $3F_{11}$, (10^7 cpms) were added. After 1 h of incubation, the plates were processed as described in the first screening procedure. Immobilized hybridoma culture supernatants that failed to bind europium-labelled anti-EG in the presence of EG were classified as β-type, and the wells that gave positive results were classified as α-type.

Screening assay 4: Identification of a matched pair of α-type and β-type secreting hybridomas. The idiometric assay is based on the use of three matched antibodies, the primary antibody, α-type and β-type. In particular, the α-type that is selected in the assay will not bind to the primary antibody-β-type complex due to epitope proximity. In order to choose the matched α-type–β-type pair the two strong α-type hybridomas that were identified in the third screening procedure were added (50 μl/well) in quadruplet to anti-mouse IgG coated microtiter wells containing 150 μl of assay buffer. The plates were incubated for 1 h, washed and blocked as described in the first screening procedure. Subsequently, to one set of duplicates, 200 μl of assay buffer containing europium-labelled anti-EG, clone $3F_{11}$, (10^7 cps/ml) were added. To the other set of duplicates, 200 μl of assay buffer containing culture supernatants secreting the strong β-typic antibodies identified in screening assay 3 and europium-labelled anti-EG (10^7cps) were added. The plates were processed as described in the first screening procedure. The α-type anti-idiotypic antibody that failed to bind the europium-labelled anti-EG antibody in the presence of the β-type was selected for the idiometric assay since its epitope was in close proximity to the epitope recognized by the β-type anti-idiotypic antibody.

Propagation of anti-idiotypic antibodies

β-type (clone # $22B_4$) and α-type (clone # $10F_{10}$) anti-idiotypic antibodies against anti-EG (clone # $3F_{11}$) were propagated *in vivo* as ascites in the peritoneum of pristane primed CD_2 male mice (age: 2 months). IgG fractions were isolated from ascites by chromatography on Sepharose-Protein A [19].

Biotinylation of anti-idiotypic antibodies

The IgG fraction of the α-type anti-idiotypic antibody, clone $10F_{10}$, was biotinylated with biotin-ε-aminocaproyl-γ-butyryl-*N*-hydroxysuccinimide ester using conditions described previously [19].

Idiometric assay for urinary EG

In order to measure urinary EG we used a variant of the idiometric assay format (see Fig. 2) in which the europium-labelled primary antibody is sequentially reacted in streptavidin coated plates with standards or samples, the β-type and finally with the biotinylated α-type. At the end of the incubation, the plates are then processed for time resolved fluorescence. For this purpose we prepared EG standards (0–10 nmol/l) in assay buffer and urine samples diluted 1:50 in assay buffer. We added 20 μl of standard or diluted urine sample in duplicate to the streptavidin coated microtiter wells containing 100 μl of a 1:200 dilution of europium-labelled anti-EG in assay buffer. The binding reaction was performed at room temperature for 30 min with the shaker. 50 μl of assay buffer containing a suitable (100-fold) dilution of the β-type (clone # $22B_4$) derived from ascites was added to each well. After 30 min, we added 100 μl of assay buffer containing the biotinylated α-type (100 ng/well; clone $10F_{10}$) and continued the binding reaction for another 60 min. The strips were then washed six times, and 200 μl of enhancement solution was added to each well. The strips were agitated on the shaker for 10 min and afterwards fluorescence was measured with the Arcus time-resolved fluorometer. The unknown values were derived from calibration curves (signal vs concentration of EG, nmol/l), and multiplied by the dilution factor, 50.

Urine samples

Early morning urine samples were collected daily throughout one complete menstrual cycle from 3 non-pregnant women for use in validation studies.

RESULTS

Screening assays

The fusion experiment yielded 23 hybridoma that secreted antibodies against anti-EG (clone $3F_{11}$). Of these, eight demonstrated strong allotypic activity, seven strong β-typic activity, four weak β-typic activity and four strong α-typic activity.

Representative screening results for five hybridomas using screening assays 1, 2, 3 and 4 are shown in Fig. 3(A and B). All the hybridomas shown in Fig. 3(A) gave positive results when tested for their ability to bind europium-labelled anti-EG (screening assay 1). Of the five hybridomas shown in Fig. 3(A), only one, hybridoma # 16B8, was able to bind europium-labelled anti-EG as well as europium-labelled antibody of the same isotype as anti-EG (clone $4A_9$), indicating that hybridoma #16B8 is a xenotypic antibody (screening assay 2). Of the remaining four hybridomas, two hybridomas (# $6C_8$ and # $22B_4$) competed with excess EG for binding europium labelled anti EG, indicating that these hybridomas

Table 1. Precision of EG measurement by idiometric assay

	Intraassay mean			Interassay mean		
Urine pools	*n*	EG (nmol/l)	CV (%)	*n*	EG (nmol/l)	CV (%)
Low	12	59.9	7.1	6	64.5	10.2
Medium	12	135	5.5	6	129	6.1
High	12	256	4.2	6	267	2.6

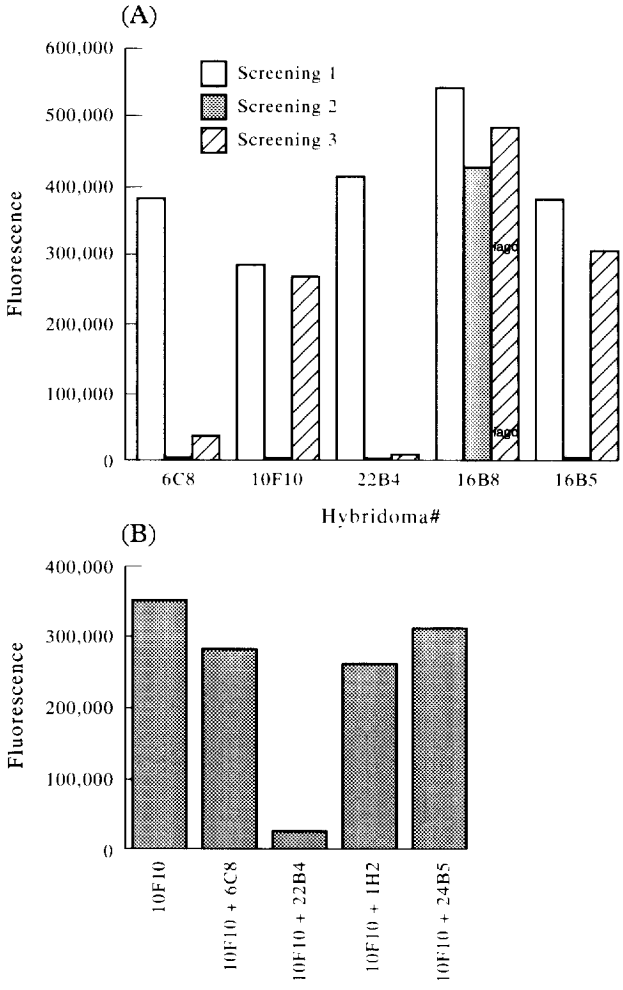

Fig. 3. (A) Screening assay 1: immunoglobulin detection. Counts obtained when the hybridomas were immobilized on anti-mouse Ig coated plates and tested for their ability to bind europium-labelled anti-EG. Screening assay 2: anti-xenotypic antibody detection. Counts obtained when the positive hybridomas from screening assay 1 were immobilized on anti-mouse plates and screened for their ability to bind a europium-labelled irrelevant antibody (clone $4A_9$) of the same isotype as anti-EG clone $3F_{11}$. Note that the hybridoma #$16B_8$ was able to bind europium-labelled clone $3F_{11}$ as well as europium-labelled clone $4A_9$, indicating the this hybridoma is an anti-xenotypic antibody. Screening assay 3: analyte sensitivity. The hybridomas that gave a negative result in screening assay 2 were immobilized on anti-mouse plates and tested to bind europium-labelled anti-EG (clone 3F11) in the presence of excess EG (100 ng/well). Note that hybridomas # $6C_8$ and # $22B_4$ failed to bind europium-labelled anti-EG in the presence of excess EG, indicating that these are β-typic anti-idiotypic antibodies while hybridomas # $10F_{10}$ and # $16B_5$ gave positive results indicating that these hybridomas are α-typic anti-idiotypic antibodies. For further details see Materials and Methods. (B) Screening assay 4: β-type sensitivity. The α-type hybridoma # $10F_{10}$ identified in screening assay 3 was screened for epitope proximity to the paratope by testing its ability to bind to the europium-labelled primary antibody in the presence of the strong β-types. Note that hybridoma $22B_4$ failed to bind europium-labelled anti-EG in the presence of the α-type, while the signal obtained with the β-typic hybridomas # $6C_8$, # $1H_2$ and # $24B_5$ was significant. These results indicate that the epitopes of hybridoma # $22B_4$ and # $10F_{10}$ are close to each other. For further details see Materials and Methods.

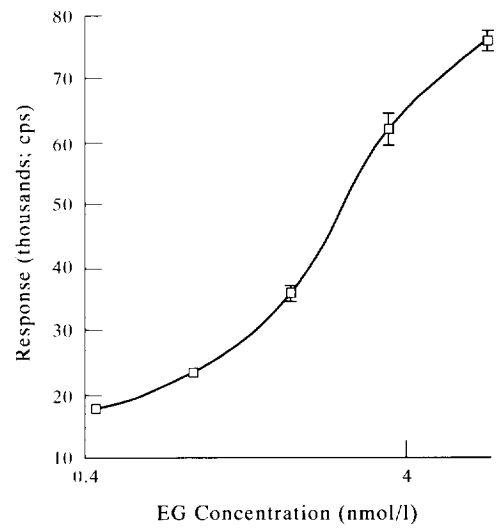

Fig. 4. Calibration curves (n=8, mean ± SD) for EG using the idiometric assay principle.

were β-type. The other two hybridomas, # $10F_{10}$ and # $16B_5$ did not compete with excess EG, indicating that these two hybridomas were α-type (Screening Assay 3).

When the various β-type hybridomas were tested for their ability to bind europium-labelled anti-EG in the presence of the α-type, only the pair of anti-idiotypic antibodies consisting of immobilized α-type $10F_{10}$ and β-type $22B_4$ gave a negligible signal (screening assay 4). Figure 3(B) shows the screening results obtained when the α-type $10F_{10}$ was immobilized and its ability to bind europium-labelled anti-EG was tested in the presence of various β-type anti-idiotypic antibodies.

Calibration curve

Calibration curves for EG were generated using the conditions described in Materials and Methods and the scheme shown in Fig. 2. Sensitivity, defined as the minimum detectable dose of EG that could be distinguished from zero (mean ± SD) was calculated from six consecutive calibration curves prepared in duplicate (see Fig. 4). The mean value derived from the curve used in the idiometric assay was 0.4 nmol/l.

Specificity

The primary anti-EG antibody, clone $3F_{11}$, cross reacted 100% with EG and showed minimal cross-reaction (<0.1%) with oestrone, oestrone-3-sulfate, oestriol-3-glucuronide, oestradiol, oestriol and oestradiol-3-β-glucuronide when using the idiometric assay conditions.

Precision

Intra-assay and inter-assay variation were estimated by measuring EG levels in duplicate in three urine pools in six consecutive assays. The results are shown in Table 1.

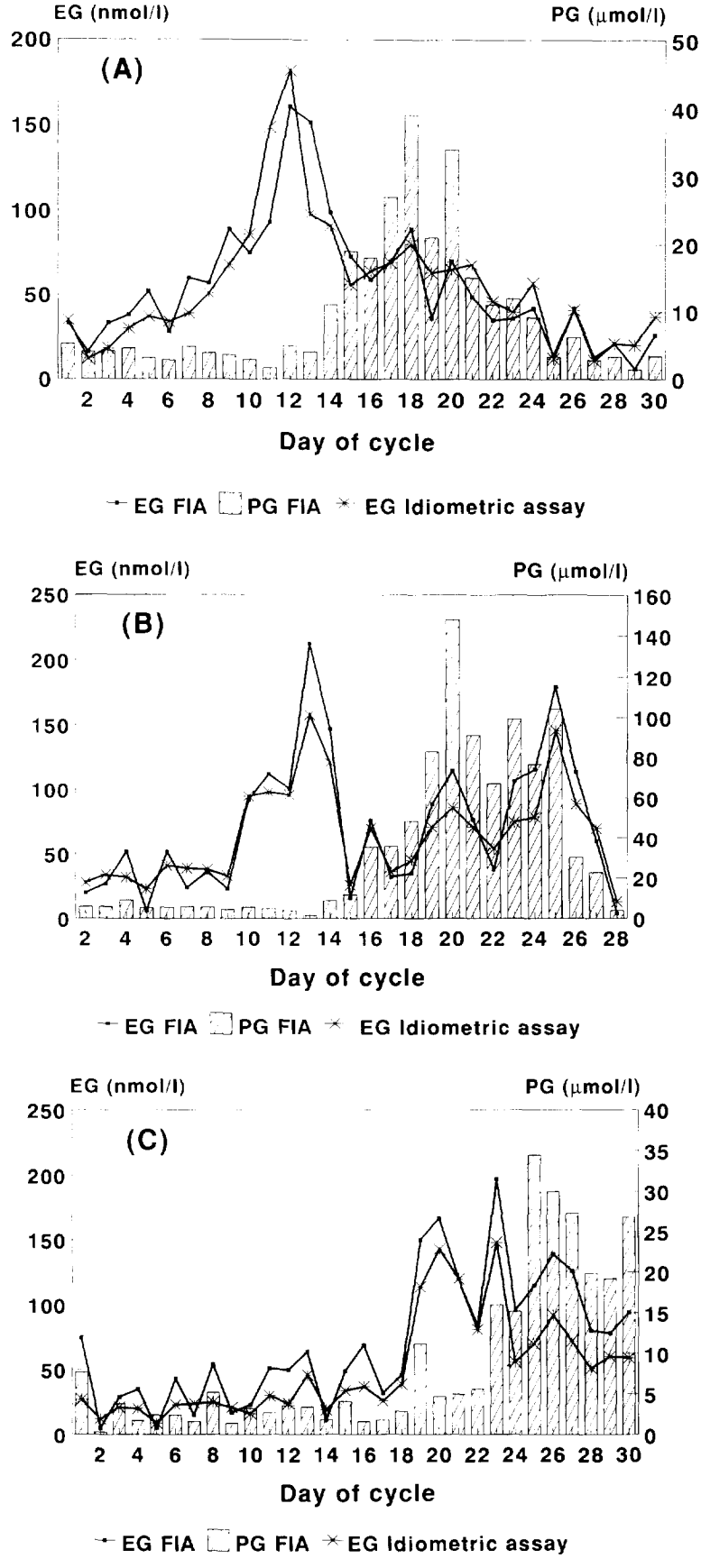

Fig. 5. The measurement of EG in samples of early morning urine collected throughout the human menstrual cycle from 3 normal non-pregnant women (shown as A, B and C) as measured by the present idiometric assay and the competitive fluorometric assay (FIA). In addition to the EG levels the urinary pregnanediol-3α-glucuronide (PG) levels were also determined by a competitive fluoroimmunoassay using the conditions described in ref. [5].

Correlation with competitive immunoassay

The concentration of urinary EG in 88 samples as determined by idiometric assay (y) and as measured by a competitive fluorescent immunoassay FIA (x) showed good correlation characteristics. The linear regression equation was $y = 0.723x + 8.98$ with a correlation of $r = 0.926$. Urinary EG levels throughout a human menstrual cycle as measured by the idiometric assay and by the competitive fluoroimmunoassay are shown in Fig. 5(A, B and C).

DISCUSSION

This paper describes current progress in the generation, characterization and application of anti-idiotypic antibodies in the development of non-competitive immunoassays for the measurement of small molecular weight analytes. The procedure, which we have termed idiometric assay [11], involves the use of two types of anti-idiotypic antibody, namely an analyte sensitive antibody (i.e. the β-type) and the analyte insensitive antibody (i.e. the α-type; see Fig. 1). In addition, the α-type selected for idiometric assay will not bind to the primary anti-analyte antibody in the presence of the β-type due to epitope proximity [see Fig. 3(B)]. Consequently, the complex that is immobilized and ultimately detected only involves the primary antibody bound to the analyte and not to the β-type. In other words, the signal generated is directly proportional to the concentration of analyte. In this report, we have described various monoclonal antibody screening experiments (see Fig. 3) that we did following the immunization of CD_2 mice with anti-EG–KLH conjugate. From the results obtained, we were able to select a matching pair of β-type (clone # $22B_4$) and α-type (clone # $10F_{10}$) anti-idiotypic antibodies that has permitted the development of a non-competitive assay for the determination of EG in samples of diluted urine. The non-competitive character of the procedure is illustrated by the calibration curves shown in Fig. 4. In addition, the method demonstrates good sensitivity and precision (see Table 1) and has acceptable specificity when compared with an alternative competitive immunoassay (see Fig. 5).

In the current format, the idiometric assay involves the sequential addition of primary antibody, the β-type and α-type. This configuration may be suitable for dipstick technology where the various antibodies can be situated in an immunostrip of absorbent material. In this format the analyte would be drawn into the device by capillary action to bind to the labelled primary antibody. Subsequently, the labelled complex would be drawn into contact with the β-type which would block the unoccupied binding sites. The mixture would then pass over immobilized α-type which would trap only the analyte–primary antibody complex and not the β-type–primary antibody complex.

Currently, we are investigating the suitability of this approach for fertility regulation in the home environment.

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