Measurement of Estrone-3-glucuronide in Urine by Rapid, Homogeneous Time-Resolved Fluoroimmunoassay

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We describe a liquid-phase nonseparation time-resolved fluorescence immunoassay for measuring estrone-3-glucuronide in undiluted urine. The sensitivity, specificity, and accuracy are similar to those for a conventional separation fluoroimmunoassay or radioimmunoassay, but the speed, convenience, precision, reliability, and clinical utility of the new method are more advantageous. The labeled antigen, a fluorescent europium chelate covalently linked to estrone-3-glucuronide, is incubated for 10 min with a limited concentration of polyclonal or monoclonal antibodies to estrone-3-glucuronyl-6-bovine serum albumin and 10 μL of standard or sample (undiluted urine) in microtiter wells. The fluorescence emanating from the antibody-free label, which is proportional to the concentration of estrone-3-glucuronide in the standard or sample, is then measured in a time-resolved fluorometer. The method is useful for monitoring ovarian function in women.

Additional Keyphrases: nonradioisotopic immunoassay · fertility studies · nonseparation assay · menstrual cycle

Numerous methods have been devised for investigating ovarian function and monitoring and predicting variations in potential fertility in women (1). In particular, results from studies of ovarian function and steroid metabolism suggest that measurement of a urinary metabolite of estradiol, estrone-3-glucuronide (E-3-G), might be used to mark the start of the fertile period (2). Accordingly, radioimmunoassay (RIA) methods have been developed to measure E-3-G in diluted urine (3) and to investigate the usefulness of this assay in delineating the fertile period and predicting ovulation (4, 5).

One of the essential prerequisites of RIA, however, has been the necessity to physically isolate the antibody-bound and free labeled ligand before signal detection. With the introduction of nonradioisotopic immunoassays, homogeneous or nonseparation immunoassays have been developed, based on modulation of the signal emanating from the antibody-bound labeled ligand. Mechanisms for this modulation include: (a) signal enhancement (6), (b) signal quenching (7), and (c) energy-transfer reactions (8).

Labels used in developing homogeneous immunoassays have included enzymes (9), chemiluminophores (10), fluorophores (11), and particles (12). The major limitation to most of these assays has been nonspecific interferences from contaminants in biological materials, assay buffers, reagents, and plastics used in the assays. Consequently, these methods have been applied to measuring analytes that are present only in relatively high concentration.

The fluorescence half-life of complex chelates of certain lanthanide elements—e.g., europium (Eu) and terbium (Tb)—is as much as six orders of magnitude longer than conventional fluorescent labels (13). Consequently, the emission from lanthanide chelates can be distinguished from background fluorescence (which has a short decay half-life) by using a time-resolved fluorometer with appropriate delay, counting, and cycle times. In particular, this approach should be ideal for more-sensitive homogeneous immunoassays by minimizing interference from fluorescent contaminants.

Here we describe the development and the evaluation of a rapid, homogeneous time-resolved fluorescence immunoassay (FIA) for measuring E-3-G in undiluted urine. This assay is derived from the synthesis of a novel europium chelate that is fluorescent in aqueous solution, thereby obviating the use of an enhancement reagent, as in conventional time-resolved FIA. The principle of the assay is as follows: the fluorescence is quenched when the labeled ligand is bound to specific antibodies; consequently, after the antibody–antigen binding reaction, the measured fluorescence emanates mainly from the antibody-free label. An increase in the concentration of competing analyte increases the concentration of the antibody-free labeled derivative and thus also the fluorescence emitted. Therefore, the need to separate antibody-bound and free fractions is obviated.

We have assessed the method for sensitivity, specificity, and precision. In addition, we compare the values for E-3-G determined by the FIA with those by a solid-phase separation FIA and by a liquid-phase RIA in which a tritiated antigen is used.

Materials and Methods

Materials

Reagents. Steroids, Tris, bovine serum albumin (BSA, Fraction V), bovine gamma globulin, Tween 20 surfactant, and diethylstilbestrol pentaacetic acid were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Solvents and chemicals used in the synthesis of the labeled antigen were analytical-grade reagents.

Buffers. Two buffers were used: (a) carbonate buffer (coating buffer; 50 mmol/L, pH 9.6), prepared by dissolving 2.93 g of anhydrous sodium hydrogen carbonate, 1.59 g of anhydrous sodium carbonate, and 0.2 g of sodium azide in 1 L of doubly distilled water; and (b) Tris HCl buffer (FIA buffer) prepared by dissolving 6 g (50 mmol) of Tris in 1 L of doubly distilled water containing 5 g of BSA, 0.5 g of bovine gamma globulin, 20 μmol of diethylstilbestrol pentaacetic acid, 0.5 mL of Tween 20, 9 g of NaCl, 0.5 g of Na2SO4, and enough HCl to adjust the pH to 7.75.

Enhancement solution for the separation FIA (obtained from Wallac Oy, Turku, Finland) contained: 1 g of Triton X-100 surfactant, 6.8 mmol of potassium hydrogen phthalate,
100 mmol of acetic acid, 50 μmol of tri-n-octylphosphine oxide, and 15 μmol of 2-naphthyl trifluoroacetone in 1 L of doubly distilled water. Wash solution (pH 7.76) for the separation FIA was prepared by dissolving 0.6 g of Tris in 1 L of doubly distilled water containing 50 μL of Tween 20, 0.9 g of NaCl, 0.5 g of Na₂SO₄, and HCl (to adjust the pH).

Antibodies. Rabbit polyclonal antibodies to estrone-3-glucuronyl-6-BSA were kindly donated by Mr. Saulat Sufi, Endocrine Unit, Chelsea Hospital for Women, London SW1, U.K. Monoclonal antibodies to estrone-3-glucuronyl-6-BSA (clones: 156B3, 3F11, A7, and 73ID6) were prepared by procedures previously reported (4).

Sample Collection, Storage, and Dilution

Daily specimens of first morning urine, collected by a healthy, nonpregnant female volunteer throughout her complete menstrual cycle, were stored at 4 °C until analysis. The samples were diluted 20-fold in buffer for the RIA but were used undiluted in the FIA.

Procedures

Preparation of labeled antigen. We prepared methyl(2,3,4-tri-O-acetyl-1-bromo-1-deoxy-alpha-D-glucopyran) uronate from D-glucurono-6,3-lactone (15), using base-catalyzed esterification, acid-catalyzed acetylation, and HBr in acetic acid for bromination. H nuclear magnetic resonance of the synthesized material confirmed its structure. We coupled the glucuronyl residue to estrone by the Koenigs-Knorr reaction in anhydrous toluene, with cadmium carbonate (Aldrich Chemical Co., Milwaukee, WI; cat. no. 22, 950-4) as a catalyst (16). Simultaneously the protecting groups were removed by treatment with methanolic sodium hydroxide and the desired estrone-3-glucuronide was prepared by acidification. Again, results of H nuclear magnetic resonance confirmed the structure.

The N-hydroxysuccinimide ester of estrone-3-glucuronide, prepared by a modification of the method of Anderson et al. (17), was coupled to a europium chelate (W 1174, Wallac Oy) in dioxane/water solution. The estrone derivatives in all steps were purified by thin-layer chromatography with suitable eluents. The fluorescent derivative has shown no significant deterioration during eight months.

Homogeneous FIA. E-3-G standards were prepared in undiluted urine obtained from a male volunteer (E-3-G concentration: 10 nmol/L) to cover the final concentration range 10 to 458 nmol/L. We added 10 μL of standard or sample (undiluted urine) to microtiter wells, in duplicate, then added 100 μL of FIA buffer containing an appropriate concentration of polyclonal or monoclonal antibodies to estrone-3-glucuronyl-BSA, and 100 μL of FIA buffer containing 2 ng of estrone-3-glucuronide-europium conjugate. We allowed the antibody–antigen binding reaction to proceed at room temperature for 10 min, using an automatic plate shaker. Subsequently, we measured the fluorescence with an Arcus time-resolved fluorometer (Wallac Oy). The unknown values were determined by comparison with calibration curves (signal vs. concentration of E-3-G, nmol/L).

Separation FIA. Affinity-purified donkey anti-rabbit IgG, 0.7 mg/mL, was diluted 200-fold in coating buffer. We added 200 μL (700 ng) of IgG to the wells of polystyrene microtiter stripe (8 × 12 well Microstrip no. 9502 107; Labsystems U.K. Ltd., Enfield, Middlesex, U.K.). After an overnight incubation at 4 °C, the coating buffer was aspirated and discarded, and the strips were washed and aspirated twice with wash solution. We then covered the strips and stored them dry at 4 °C until required.

E-3-G standards, 0 to 448 nmol/L, were prepared in FIA buffer. We added 20 μL of these standards or sample (undiluted urine) to the coated microtiter wells, in duplicate, then added 100 μL of FIA buffer containing rabbit polyclonal antibodies to estrone-3-glucuronyl–BSA (diluted 1:10 000 in FIA buffer) and 100 μL of estrone-3-glucuronyl–europium conjugate (2 ng/mL, in FIA buffer). The antibody–antigen binding reaction proceeded at room temperature for 1 h, with samples being shaken on an automatic plate shaker. We then washed the solid phase six times. After adding 200 μL of enhancement solution to each well, we agitated the strips on the shaker for 15 min, then measured the fluorescence with the Arcus time-resolved fluorometer. The unknown values were determined as above.

Radioimmunoassay. We measured E-3-G in diluted urine according to the method of Collins et al. (4), using a tritiated antigen and dextran-coated charcoal to separate the antibody-bound and free labeled ligand.

Results

Calibration curves. Figure 1 shows calibration curves for E-3-G (range: 10–916 nmol/L) obtained with the homogeneous FIA and different dilutions of polyclonal or monoclonal (clone 3F11) antibodies. From these results, we decided to use the polyclonal antibodies, diluted 10 000-fold, or 1000-fold-diluted monoclonal antibodies in FIA buffer. Figure 2 shows calibration curves for E-3-G (range: 10–458 nmol/L) obtained at optimal concentrations with five different antibodies. The precision profiles obtained at these optimal
Table 1. Specificity of Rabbit Polyclonal and Four Monoclonal Antibodies in Measurements of E-3-G by Homogeneous FIA

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Discussion

The application of immunoassay to facilitate numerous sensitive and specific methods for quantifying haptens and high-molecular-mass analytes has generated an increasing demand for a wider availability of these procedures, to accommodate effective treatment and monitoring of various normal or pathological conditions. For example, the investigation of human fertility, the monitoring and treatment of infertility, and the introduction of in-vitro fertilization techniques all require rapid assessment of hormonal status. The use of RIA, however, restricts the technique to those laboratories licensed to handle radioactivity. In addition, the need to separate the antibody-bound and free labeled ligand in RIA may make the procedures more time-consuming and expensive and, in some cases, inappropriate for rapid screening.

In recent years, nonradioisotopic procedures have been developed that are as sensitive and specific as the equivalent RIAs (19, 20). However, they have been criticized as more complicated, time-consuming, and less precise than the equivalent RIAs. Nevertheless, their introduction has enabled the development of homogeneous immunoassays.

Several homogeneous immunoassays have been developed in which an enzyme is used as the label (9), its activity being inhibited when the enzyme–ligand conjugate is bound to the antibody. This approach has been successfully commercialized (xma; Syva Corp., Palo Alto, CA). In addition, homogeneous chemiluminescence immunoassays have been developed with isoluminol as the label, which involve the enhancement or inhibition of the signal when the ligand conjugate is bound to the antibody (10). In perhaps the most successful commercial homogeneous procedure, fluorescein is used as the label in fluorescence polarization immunoassay (11), which involves the maintenance of polarization when the labeled ligand is bound to the antibody. The free rotation of the antibody-free fraction decreases the polarized emitted light (TDx system: Abbott Diagnostics, Abbott Park, IL).

The advantages of non-separation immunoassay include: (a) simplicity, (b) speed, (c) increased precision, and (d) the potential for total automation. Nevertheless, most of the non-separation immunoassays developed to date have been subject to nonspecific interference from components of biological materials, assay buffers, reagents, and plastics. Consequently, the overall assay sensitivity of these methods has limited their use to the measurement of analytes found in relatively high concentrations.

The introduction of separation time-resolved FIAs has been highly successful and has enabled the development and the routine use of a wide range of assays for the measurement of haptens and proteins with appropriate sensitivity, specificity, and precision (21). In addition, it has been argued that the principle of time-resolved fluorescence offers the greatest potential for the development of more sensitive separation immunoassays (22) and homogeneous immunoassays (21).
The free europium forms a highly fluorescent secondary chelate with the diketone in a micellar non-aqueous environment. This extra step limits the potential sensitivity of the assays by allowing a degree of contamination by free europium from the environment and lowering the possible signal-to-noise ratio. In addition, the development of nonseparation procedures has been precluded.

The second type of assay involves the use of a chelator saturated with europium as a label (23). After separation and the removal of the excess material, the relatively unstable europium chelate is excited by a laser and the fluorescence is measured directly from the solid-phase. Because this lanthanide chelate is stabilized by the presence of excess europium, the development of nonseparation FIA would appear to be impossible.

Recently, Hemmila et al. (24) described preliminary results obtained with a novel fluorescent chelate as a label in a homogeneous FIA for the measurement of thyroxin in serum. This approach has enabled the development of a second generation of time-resolved FIA.s. In this report, we have described the use of a fluorescent estrone-3-glucuronyl-europium derivative to develop a simple, rapid, and precise homogeneous FIA for measuring urinary E-3-G. In terms of specificity and accuracy, the characteristics of the assay are comparable to a separation FIA or RIA with use of polyclonal antibodies. This is illustrated by the measurement of E-3-G in samples of first morning urine collected from a normal, healthy menstruating woman. In terms of speed, precision, reliability, and clinical utility, however, the homogeneous FIA has several advantages. In addition, the rapid counting time and data handling facilities of the Arcus time-resolved fluorometer make the assay extremely simple and convenient to perform.

This rapid and precise method for measuring E-3-G in urine will facilitate effective treatment and management of infertility. In addition, we are currently assessing the usefulness of the procedure to delineate the fertile period in women, the prediction of ovulation, and the monitoring of patients undergoing ovarian stimulation regimes before in vitro fertilization.

References


