Time-Resolved Immunofluorometric Assay of 17β-Hydroxysteroid Dehydrogenase in Plasma

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We describe a time-resolved immunofluorometric assay (TR-IFMA) for human 17β-hydroxysteroid dehydrogenase (17HSD) in which antibody-coated microtiter strip wells and europium chelate-labeled polyclonal antibodies are used. In preparing the label, a polyclonal antibody is affinity-purified and derivatized with diethylenetriaminepentaaetic acid. With this derivative, five to eight europium ions can be combined with one antibody molecule without decreasing the antibody's immunoreactivity. The minimum detectable concentration of 17HSD is 0.13 μg/L; the intra- and interassay CVs are <8% and <15%, respectively, for concentrations between 0.3 and 100 μg/L. There is no difference between the concentrations of 17HSD in plasma specimens taken during the proliferative and luteal phases of the menstrual cycle, the measured mean concentration being 0.22 μg/L. We found no correlation between plasma 17HSD and progesterone concentrations. The plasma concentrations of 17HSD increase during pregnancy, the mean concentrations being 1.5, 4.4, and 12.5 μg/L, during the first, second, and third trimesters of pregnancy, respectively. In the specimens from 18 men, the mean concentration was 0.18 μg/L. In six plasma specimens from patients with endometrial adenocarcinoma, the mean concentration was 0.20 μg/L. Pre-analytical aspects are important in the assay of 17HSD because of the lability of the enzyme protein. Preferably, blood should be sampled into EDTA-containing tubes, plasma should be separated within 15 min, and glycerol must be added without delay to a final volume of 200 mL.

Additional Keyphrases: pregnancy · menstrual cycle · glycerol as preservative · sample handling · endometrial adenocarcinoma

17β-Hydroxysteroid dehydrogenase (17HSD; EC 1.1.1.62) is present in steroid-forming tissues (1, 2) and catalyzes the interconversions of 17β-hydroxysteroids and 17-oxosteroids. It is also present in certain target tissues for steroid hormone action such as the endometrium (3), in which it is thought to help regulate the exposure of the tissue to estrogen by converting estradiol into the less potent estrogen, estrone (4). In the endometrium, the activity of this enzyme is regulated by progestins (1, 2, 5–7).

17HSD has been purified to homogeneity in this (8) and other laboratories (9, 10) by using placental tissue as the starting material. Furthermore, we have raised polyclonal antibodies against the purified protein (8) and sequenced the cDNA of human placental 17HSD (11).

Circulating concentrations of 17HSD possibly reflect the functional status of steroid-forming or progesterone-regulated tissues. During pregnancy, blood concentrations of this enzyme might therefore reflect the state of the placenta, known to be a rich source of this enzyme (8–10). To this end, the enzymatic activity of 17HSD has been measured in the serum of pregnant women (12, 13) and in umbilical cord serum (13). However, the lability of the enzyme activity has created problems in kinetic assays of the enzyme in serum (12). Time-resolved immunofluorometric assays (TR-IFMA) offer an attractive method for quantifying circulating analytes expected to be present in low concentrations (14–16).

In this study, we used an inexpensive europium (Eu)-labeled method (17) to prepare a diethylenetriaminepentaaetic acid (DTPA) derivative of our polyclonal antibody (8) and developed a TR-IFMA for measuring circulating 17HSD.

Materials and Methods

Materials

Antigen. We purified 17HSD to apparent homogeneity from human term placental tissue, as described previously (8).

Antibody production and purification. By using the highly purified placental 17HSD as an immunogen, we raised polyclonal antibodies in rabbits. We used two of these antibodies to construct an immunometric assay. The equilibrium dissociation constants ($K_d$) of the antibodies were $3.7 \times 10^{-11}$ and $4.9 \times 10^{-11}$ mol/L, as determined by Scatchard analysis (18). The antibodies were purified by affinity chromatography on a Sepharose–Protein A column (Pharmacia, Uppsala, Sweden). We used the antibody with greater affinity to prepare the labeled derivative; the other antibody was immobilized on solid phase.

Preparation of Eu-Labeled Antibody

Eu-labeling and purification of the labeled antibody were carried out as described previously (17). We conjugated the affinity-purified antibody with the bicyclic anhydride of DTPA (6dTPAA), using a 6dTPAA/IgG molar ratio of 40/1 in the conjugation reaction.
Coating of Microtiter Strips

Polystyrene microtiter strips forming 96-well plates (Labsystems Oy, Helsinki, Finland) were coated at room temperature with 200 µL of the coating solution for 48 h. The solution consisted of 4 µg of the IgG fraction of the antibody per milliliter. The antibody was treated with 0.5 mol/L sodium citrate buffer, pH 2.5, for 30 min at room temperature and diluted in 0.1 mol/L sodium phosphate buffer, pH 7.5. After the coating, we rinsed the wells three times with 5 mmol/L Tris·HCl buffer, pH 7.75, containing 150 mmol of sodium chloride and 5 mg of Tween 20 per liter and the bacteriostatic agent Germall II (Chemag, Frankfurt/Main, F.R.G.). Finally, we incubated the plates at room temperature in a 50 mmol/L Tris·HCl buffer, pH 7.5, containing, per liter, 160 mmol of sodium chloride, 1 g of sodium azide, and 50 g of sorbitol; after 30 min, we decanted the liquid and dried the plates at room temperature. The plates were sealed in plastic bags and stored at 4 °C, where they were stable for at least six months.

17HSD TR-IFMA

The assay buffer we used was Tris·HCl (50 mmol/L, pH 7.5), containing, per liter, 9 g of NaCl, 5 g of bovine serum albumin, 0.5 g of bovine γ-globulin, 0.1 g of Tween 40, and 20 µmol of DTPA. The wash solution was the same as described above for rinsing the coated microtiter strips. We used enhancement solution as supplied by the manufacturer (Pharmacia, Turku, Finland).

We used a Model 1230 Arcus fluorometer, a Model 1296–024 Platewash automated aspirating/washing device, and a Model 1292 Rack Shaker (all from Wallac, Turku, Finland), compatible with the strip wells.

We constructed the standard curve by using concentrations of 0, 0.3, 1, 3, 10, 30, and 100 µg/L of purified 17HSD in inactivated charcoal-treated human serum containing, per liter, 200 mL of glycerol.

Duplicate 100-µL aliquots of each of the standard and plasma samples were pipetted into the coated wells. We then added 100 µL of the assay buffer to each well and incubated the contents for 4 h at room temperature on an automated shaker. We rinsed the wells six times with wash solution, using the Platewash device. The anti-17HSD–Eu-tracer in assay buffer (15 × 10⁶ counts/s per 200 µL), diluted within 1 h before use, was added to the wells, then incubated for 3 h on an automated shaker at room temperature, and again washed six times with wash solution. Then we added 200 µL of enhancement solution to each well. The wells were shaken for 5–10 min and left for ≥30 min before we measured the fluorescence (1 s/well).

Blood Specimens

Blood specimens were sampled from apparently healthy men (n = 18, ages 29 to 55 years), normal menstruating women (n = 25), women with endometrial adenocarcinoma (n = 6), and 50 normal pregnant women.

Blood was collected into EDTA Vacutainer Tubes (Becton Dickinson, Meylan Cédex, France). Plasma was separated within 15 min of collection and glycerol was added without delay, to give a concentration of 200 mL/L.

Results

Antibodies and Labeling

The antigen and the antibody with the Kᵅ of 3.7 × 10⁻¹¹ mol/L have been described in detail already (8). In the present study, we used an additional purified polyclonal antibody, raised against the purified 17HSD, as a capture antibody in an immunometric assay.

The antibody with the Kᵅ of 3.7 × 10⁻¹¹ mol/L was purified, derivatized with cDTPAA, and labeled with Eu. Five to eight Eu atoms could be combined with one IgG molecule.

Analytical Variables

A standard curve for the TR-IFMA of 17HSD is shown in Figure 1.

Sensitivity. The lowest detectable concentration of 17HSD, defined as the concentration corresponding to the counts per second 3 SD above the mean of 18 replicate determinations of the zero ligand concentration, was calculated as 0.13 µg/L.

Analytical recovery. We performed the recovery tests by adding known amounts of the purified analyte to blood plasma. We recovered 83–113% of 17HSD added in the concentration range of 3.1–33.4 µg/L.

Linearity. We studied the effect of serial dilutions with three plasma samples, containing 17HSD at 8.7, 14.3, and 18.4 µg/L. The respective linear regression equations for the dilution curves were y = 0.99x + 0.05, y = 0.99x + 0.1, and y = 1.03x – 0.9.

Precision. We carried out the precision tests with plasma specimens from pregnant women. To determine within-run variability (CV), we made six duplicate measurements of low (2.8 µg/L), medium (10.5 µg/L), and high (25.2 µg/L) concentrations of 17HSD. The mean CVs for these measurements were 11.4%, 6.7%, and

![Fig. 1. Linearized (log-log) standard curve and precision profile for the TR-IFMA of 17HSD. The mean and SD for six replicate assays are shown on each standard point](image-url)
5.4%, respectively. We calculated between-run variability on the basis of duplicate measurements of plasma specimens with 17HSD concentrations of 2.1, 8.5, and 16.9 μg/L, carried out on 10 occasions. The mean CVs were 14.7% at low concentration, 14.3% at medium concentration, and 6.3% at high concentration.

*Stability tests.* The known lability of the enzyme in kinetic assays was also evident in our immunometric assay. When stored at room temperature for only 24 h, ~60% of the immunoreactivity was lost; this loss increased to ~80% after 48 h. The corresponding losses were 35% and 62% when the specimens were stored at 4°C. The stability of 17HSD in serum could be retained by adding glycerol, 200 mL/L final concentration, to the plasma and by freezing specimens within 2 h at -70°C. Under these conditions, the enzyme was stable for months.

Plasma Concentrations of 17HSD

The mean plasma concentration of 17HSD in the apparently healthy men examined was 0.18 μg/L (range 0.0–0.48 μg/L). There was no apparent association of the concentrations of 17HSD with age.

The concentrations of plasma 17HSD in the specimens from pre-menopausal, apparently healthy women taking no form of hormonal contraception are shown in Table 1. There was no relation between the 17HSD concentrations and the phase of the menstrual cycle, nor with serum progesterone concentrations (data not shown). The mean 17HSD concentrations were 0.22 μg/L (range 0.02–0.84 μg/L) during the follicular phase (n = 18) and 0.22 μg/L (range 0.01–0.84 μg/L) during the luteal phase (n = 10).

During pregnancy, there was a clear increase in plasma 17HSD concentrations, as shown in Table 1.

Concentrations of plasma 17HSD were not increased in patients with endometrial adenocarcinoma (Table 1).

**Discussion**

We have developed an immunometric assay for measuring circulating concentrations of 17HSD by using a TR-IPMA. The incorporation of five to eight Eu atoms to one antibody–DTPA derivative molecule had no marked effect on the immunoreactivity of the antibody. As expected, the method is characterized by high sensitivity, owing to the high specific activity of the Eu-labeled polyclonal antibody tracer.

One problem encountered in evaluating the clinical importance of the measurements of circulating 17HSD concentrations is the lability of the enzyme. The use of different protease inhibitors did not prevent the loss of immunoreactivity. The only way we found to retain the antigenicity was to add glycerol to the samples without delay after the separation of the plasma. Perhaps this problem could be avoided by using monoclonal antibodies that interact with the more stable epitopes of the antigen.

The antigen was detectable in plasma from men, but in very low concentrations. At present, we have not ascertained the sources of this antigen. In men, 17β-HSD activity has been detected in the steroid-forming tissues (2) and the prostate (19–21).

The steroid-forming tissues could also be the source of the enzyme circulating in plasma from women. An additional source would be, at least theoretically, the endometrium. However, we found no increase in the concentrations of circulating 17HSD during the second half of the menstrual cycle. During this part of the cycle, 17HSD is present in increased concentrations in the endometrial epithelium (7, 8) because it is induced by the progesterone (5) secreted by the corpus luteum during the second half of the cycle. Our data show that these concentration changes of the enzyme in the endometrium are not reflected in its plasma concentrations and, therefore, the low amounts of circulating 17HSD may not be of endometrial origin.

The limited number of serum specimens from patients with endometrial adenocarcinoma did not reveal any changes in the plasma concentrations of 17HSD.

The increasing concentrations of 17HSD toward the end of pregnancy may reflect the increasing mass of the placental tissue and the associated increase in the turnover of placental cells. We do not know at present whether the concentrations of plasma 17HSD reflect disturbances in placental function.

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**Table 1. Concentrations of 17HSD in Plasma from Apparently Healthy Subjects and Patients with Endometrial Adenocarcinoma**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>n</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>18</td>
<td>0.18</td>
<td>0.00 – 0.48</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular phase</td>
<td>15</td>
<td>0.22</td>
<td>0.02 – 0.84</td>
</tr>
<tr>
<td>Luteal phase</td>
<td>10</td>
<td>0.22</td>
<td>0.01 – 0.84</td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First trimester</td>
<td>8</td>
<td>1.48</td>
<td>0.40 – 3.90</td>
</tr>
<tr>
<td>Second trimester</td>
<td>22</td>
<td>4.35</td>
<td>0.72 – 7.80</td>
</tr>
<tr>
<td>Third trimester</td>
<td>20</td>
<td>12.46</td>
<td>3.70 – 28.1</td>
</tr>
<tr>
<td>Endometrial adenocarcinoma, stages I–III</td>
<td>6</td>
<td>0.20</td>
<td>0.14 – 0.24</td>
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**References**


Aminoglycosides are still used extensively in the treatment of nosocomial infections with Gram-negative bacteria. However, the treatment is associated with several adverse effects. Aminoglycosides monitoring is therefore essential to prevent toxic accumulations and to reach therapeutic concentrations. A computer program, PHARMONITOR, has been developed to optimize aminoglycosides monitoring, responding to the demands of the most clinical daily situations. This program, based on a one-component open pharmacokinetic model, is developed for IBM PC-compatible computers, using D-Base III++. It can calculate t_{1/2}, V_0, C_{drug}, C_{max} and the theoretical optimal dose and interval and also evaluates the creatinine clearance. The program has been conceived to allow maximal speed, flexibility, and reliability by the use of (e.g.) a linear least-squares analysis, the possible reference to previous protocols, the extensive use of keywords to classify and recall patients according to their pathologies, the development of messages recommending maximal dose or minimal dosing interval, and increasing the safety of the analysis. We consider the program a valuable tool for adjusting aminoglycoside dosage in individuals.

Additional Keyphrases: therapeutic drug monitoring · pharmacokinetics

Since the discovery of streptomycin more than 40 years ago, aminoglycosides continue to be used extensively in the treatment of severe Gram-negative bacterial infections, particularly those acquired in the hospital. The synergistic effects of using these drugs with beta-lactams and the relatively low and reversible bacterial resistance to aminoglycosides contributed to the success of the treatment. The therapeutic activity seems to be directly dose-dependent and related to the ratio of peak serum concentration to minimal inhibitory concentration (I).

However, aminoglycosides are associated with severe adverse effects. Primarily, nephrotoxicity and irreversible ototoxicity are described as accompanying high