Radioimmunoassay of 2-Hydroxyestrone in Urine

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Catechol estrogens such as 2-OH estrone are interesting estrogen metabolites formed in several human tissues and excreted in urine. We developed and thoroughly validated a radioimmunoassay for urinary 2-OH estrone that has several advantages over published RIAs. Because we developed a relatively specific antiserum, we did not include a preliminary chromatographic step to eliminate cross-reacting urinary steroids. We hydrolyzed urinary steroid conjugates with β-glucuronidase from Helix pomatia because recoveries after acid hydrolysis were only 48.6% compared with 73.8% after enzyme hydrolysis. Published RIAs for urinary 2-OH estrone use acid hydrolysis. Our RIA measured 2-OH estrone independently of the volume of sample, and the detection limit was between 100 and 240 ng/L (10–24 pg per tube). The ED90 was 370 ng/L, and inter- and intraassay CVs for low, medium, and high concentrations were 22.5%, 22.8%, and 19.9%, and 17.4%, 14.3%, and 10.8%, respectively. Median concentrations measured in 14 controls and 33 postmenopausal patients with breast cancer were 0.96 and 1.55 μg/g creatinine, respectively, but there was considerable overlap between values from controls and patients.

Indexing Terms: catechol estrogens; menopausal status; breast cancer/ enzymatic and acid hydrolysis compared

Catechol estrogens are 2- and 4-hydroxylated metabolites of estrone and estradiol-17β formed in several mammalian tissues and excreted in urine (1–5). They have interesting pharmacological properties, including inhibition of catechol-O-methyltransferase (6, 7) and tyrosine hydroxylase (8). They may influence the secretion of luteinizing hormone (9), prolactin (10), and prostaglandins (11). The 2-OH derivatives have less affinity than the 4-OH derivatives and estradiol-17β for uterine estrogen receptors (12).

Reports that concentrations of 2-OH estrone and 16α-OH estrone in patients with endometrial or breast cancer differ from those in controls (13, 14) are interesting because concentrations of these estrogens are also altered in female cigarette smokers (15), who have a lower incidence, relative to controls, of breast or endometrial cancer (16).

Published radioimmunoassays (RIAs) for urinary 2-OH estrone used acid to hydrolyze steroid conjugates (17, 18). In our opinion, insufficient attention was given to the possible effects of acid hydrolysis on the recovery of 2-OH estrone from urine. Here, we developed and fully validated an RIA for urinary 2-OH estrone and compared the effects of acid and enzyme hydrolysis on recoveries of 2-OH estrone. We generated a relatively specific antiserum and thus did not include a preliminary chromatographic step to remove cross-reacting steroids. We determined the ability of this RIA to measure 2-OH estrone concentrations independently of the sample volume (by relation to creatinine clearance) and established normal ranges of values in 11 regularly menstruating women. We used the RIA to measure urinary 2-OH estrone in 33 women shortly after diagnosis of breast cancer and in 14 age-matched controls.

Materials and Methods

Subjects

To investigate the concentrations of 2-OH estrone during the menstrual cycle we selected 11 female control subjects, ages 21 to 40 years. They were in good health, had regular menstrual cycles (mean 29 days, SD 3 days), were nonsmokers, and were not taking oral contraceptives or other drugs. After an overnight fast they collected, during one menstrual cycle, an early-morning urine sample every second morning in 20-mL bottles that contained 600 mg of ascorbic acid. The start of each cycle was defined as 1 day after the onset of bleeding. An early-morning saliva sample for progestosterone measurement was also provided by each volunteer, beginning on day 7 and concluding on day 21 of the cycle. Urine and saliva samples were stored at −20°C. Ovulation was confirmed by enzyme immunoassay of salivary progesterone (19, 20).

Another group of control subjects were 14 postmenopausal women, ages 47 to 93 years, in good health, nonsmokers, and not taking medication. They collected one early-morning urine sample, as described above, after an overnight fast. We also studied 33 women, ages 51 to 74 years, who were diagnosed with carcinoma of breast; they collected urine samples after an overnight fast, as soon as feasible after diagnosis and before therapy commenced.

Reagents

Tetrahydrofuran was obtained from Reidel-de Haen (Seeze, Germany); β-glucuronidase (from Helix pomatia), potassium iodide, and sodium bisulfite from Sigma (Poole, Dorset, UK); glass hydrolysis tubes from Hach (Loveland, CO); and precoated silica gel 60 F_254 thin-layer chromatographic plates (20 cm × 20 cm × 0.2 mm) from Merck (Darmstadt, Germany). Proton nuclear magnetic resonance (1H-NMR) spectra were obtained with a JEOL (Tokyo, Japan) GX-270 spectrometer. 2

2 Nonstandard abbreviations: 1H-NMR, proton nuclear magnetic resonance; CMO, carboxymethylxime; and BSA, bovine serum albumin.

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80 CLINICAL CHEMISTRY, Vol. 40, No. 1, 1994
HPLC system (Milton Roy, Stone, Staffordshire, UK) used a CM400 multiple-solvent delivery system, SM400 programmable-wavelength detector, and LDC/Milton Roy CL10 integrator. A Technopak 10 (HPLC Technology, Macclesfield, Cheshire, UK) reversed-phase C18 (25 × 4.6 mm) column was used.

The standard buffer (pH 7.4) contained, per liter, 25 mmol of ascorbic acid, 3.2 mmol of EDTA Na₄ salt, 10 mmol of Na₂HPO₄/NaH₂PO₄, 150 mmol of NaCl, and 100 µL of Tween 80. This buffer was also used as the assay buffer (with the omission of Tween 80).

Carboxymethoxylamine hemihydrochloride, N,N-dimethylformamide, N-methylmorpholine, and isobutylchloroformate were obtained from Aldrich (Gillingham, Dorset, UK). Other solvents and salts were obtained from Reidel-de Haen. Steroids, including 2-OH estrone, were obtained from Steraloids (Wilton, NH).

The purity of 2-OH estrone was checked with 1H-NMR, and a stock standard solution (3.5 mmol/L in ethanol) was stored at 4°C. Working standard solutions of 2-OH estrone (139.6 µmol/L to 13.9 nmol/L) were prepared in standard buffer and stored at −70°C. Dextran-coated charcoal contained 50 mL of activated charcoal (10 g/L) and 20 mL of Dextran T70 (Sigma) (2.5 g/L) in assay buffer containing 1 g/L bovine serum albumin (BSA). This mixture, stored overnight at 4°C before use, was stable for 1 week at 4°C.

Other Materials

Preparation of 2 hydroxyestrone-17-O-carboxymethoxylxime (2-OH estrone-17-CMO). We dissolved sodium acetate trihydrate (6 mg) and carboxymethoxylamine hemihydrochloride (6 mg) in 1 drop of H₂O followed by 100 µL of methanol and added this to 10 mg of 2-OH estrone previously dissolved in 0.5 mL of methanol. We allowed the mixture to stand at room temperature (18°C) in a sealed glass container under nitrogen. The progress of the reaction was monitored by thin-layer chromatography on silica gel 60 F₂54 with use of chloroform/methanol/acetic acid (4/1/0.2 by vol) as the mobile phase. After 24 h, we added 5 mL of H₂O and extracted the reaction mixture with two 10-mL portions of ethyl acetate. The extract was dried by rotary evaporation, and the precipitate was redissolved in a mixture of deuterated chloroform/dimethyl sulfoxide (10/1 by vol) for examination by 1H-NMR. Deuterated chloroform was removed from the mixture by rotary evaporation. We dissolved the remaining dimethyl sulfoxide plus 2-OH estrone-17-CMO in 10 mL of H₂O and then extracted the product with three 10-mL portions of ethyl acetate. The ethyl acetate was again removed by rotary evaporation, and the 2-OH estrone-17-CMO was redissolved in acetonitrile. We further purified the 2-OH estrone-17-CMO in acetonitrile by HPLC, using an equimolar solution of water/acetonitrile as the mobile phase. Pooled eluate containing 2-OH estrone-17-CMO was rotary-evaporated to remove acetonitrile, and the 2-OH estrone-17-CMO was extracted from water with ethyl acetate.

Carboxymethoxylamine hemihydrochloride did not conjugate to the 2-OH or 3-OH groups of 2-OH estrone because of the presence of hydrogen atoms, which protect the oxygen atoms from forming a CMO group.

Steroid–BSA. 2-OH estrone-17-CMO was conjugated to BSA by the modified mixed anhydride method for immunization of rabbits (21, 22).

Antiserum. Rabbits were immunized by subcutaneous injections with 200 µg of steroid–BSA conjugate diluted in an equal volume of Freund’s complete adjuvant. Booster injections with 100 µg of 2-OH estrone–BSA conjugate were given at 2-month intervals in Freund’s incomplete adjuvant. The titer of the antiserum after the last immunization was 1:12 000 (in 100 µL per test). All dilutions of the antiserum were performed in assay buffer.

Radiolabeled 2-OH estrone. 2-OH[6,9-3H]Estrone, (spec. acty. 69.8 kCl/mol; Amersham International, Amersham, UK) was diluted 22-fold with assay buffer and stored at −20°C as a working stock solution. The latter solution was further diluted 500-fold, to give a final activity of −20 000 dpm per tube.

Quality-control urine. An entire early-morning urine was collected (in 30 g/L ascorbic acid) from a premenopausal control female volunteer. We divided the collection into three aliquots and added 2-OH estrone, 0, 10, or 20 mg/L, to each aliquot. The aliquots were dispensed in 1.2-mL volumes and stored at −70°C.

Extraction of 2-OH Estrone from Urine

Urinary steroids such as 2-OH estrone are conjugated to glucuronides and sulfates, which may be hydrolyzed by enzymes or acids.

Enzyme hydrolysis. Pipette 1.0 mL of urine into disposable 16 × 100 mm culture tubes and add 200 mg of NaCl. Vortex-mix the samples for four 1-min intervals. Add 5 mL of tetrahydrofuran to each tube and again vortex-mix for 4 × 1 min. Place the samples on ice and allow the phases to separate. Transfer 4.0 mL of the solvent phase to a culture tube, using a positive-displacement pipette. Evaporate the solvent to dryness at 40°C in a stream of N₂. Add to each tube 0.5 mL of β-glucuronidase (237.5 U) dissolved in assay buffer, and incubate at 37°C overnight. Vortex-mix samples for 1 min before assay.

Acid hydrolysis. We modified a published procedure (17) and proceeded as follows. Pipette 1.0 mL of urine into 16 × 100 mm screw-cap glass hydrolysis tubes. Add 100 µL of aqueous potassium iodide (1.51 mol/L), 100 µL of aqueous sodium bisulfite (2.5 g/L), and 200 µL of concentrated hydrochloric acid (12 mol/L). Seal tubes and place in a boiling water bath for 1 h. Cool tubes under cold running water and transfer 700 µL of the hydrolysate into another 16 × 100 mm test tube. Add 135 µL of sodium hydroxide (10 mol/L) and 165 µL of assay buffer, adjusted to pH 7.0. Dilute the hydrolysate 10-fold with assay buffer before RIA.

RIA of 2-OH Estrone

Optimization studies with the assay included the characterization and selection of suitable buffers and volumes.
of the various components of the assay. We determined that the following procedure performed best.

Dilute urine hydrolysates 40- and 80-fold. Add 100 µL of diluted urine hydrolysate or standard 2-OH estrone (10, 20, 40, 60, 100, and 200 pg/tube) to disposable 12 × 75 mm glass tubes. Add 100 µL of antiserum (diluted 3000-fold in assay buffer), and 200 µL of 2-OH[6,9-3H]estrone. Vortex-mix and incubate at 4°C for 4 h. Add 200 µL of freshly prepared dextran-coated charcoal to all tubes except the tube for total bound counts. Vortex-mix and incubate at 4°C for 15 min. Centrifuge all samples at 4°C (500g) for 20 min and decant 500 µL of supernate into polypropylene scintillation vials (16 × 50 mm). Add 3.0 mL of scintillant (“Ready Value”; Beckman, Fullerton, CA) to each vial. Seal all vials, vortex-mix, and count the radioactivity in a Beckman LS 1801 counter.

**Results**

**Analytical Variables**

**Standard curve and precision profile.** The standard curve (Fig. 1) represents the mean from 19 assays performed during 6 months, with triplicate determination of standards. The detection limit, defined as the concentration of 2-OH estrone required to give a B/B₀ of 2 SD below the mean counts per minute observed in the absence of cold hormone, ranged between <10 and 24 pg/test (100 to 240 ng/L) over 19 assay runs. The EDₑ₀ was 37 pg/test (370 ng/L), which is about twice as much as the detection limit of the assay. This is due to the relatively high affinity of the antiserum, which resulted in a standard curve with a steep slope. The precision profile, which represents the within-assay CV for three samples over the range of the standard curve, is also shown (Fig. 1). Table 1 shows the inter- and intra-assay variations for a low, medium, and above-normal urinary concentration of 2-OH estrone after enzyme hydrolysis of replicate samples on each assay.

**Specificity.** The specificity of the antiserum used for 2-OH estrone was assessed for a range of structurally related steroids by using the equivalent weight of all steroids (23). The antiserum demonstrated relatively high specificity for 2-OH estrone. The only other steroids that had significant cross-reactivities were 2-OH estradiol and 2-OH estradiol (17.4% and 14.4%, respectively; Table 2).

**Analytical recovery after hydrolysis.** We established the ability of the assay to accurately measure 2-OH estrone added to urine samples that contained a range of endogenous 2-OH estrone concentrations. Extraction efficiency, after either acid or enzyme hydrolysis, was assessed by adding two different concentrations of 2-OH estrone to urine samples from different subjects (Table 3). Total 2-OH estrone was measured, and the recovery of added steroid was calculated after subtracting the endogenous steroid concentration. Overall mean recoveries of added standard after acid or enzyme hydrolysis were 49.6% and 73.8%, respectively. Consequently, we used enzyme hydrolysis to release 2-OH estrone from its conjugates in urine.

**Independence of volume.** We assessed the ability of the assay to measure 2-OH estrone independently of the volume of sample after enzyme hydrolysis. After initial dilutions of hydrolysates to bring them within the range of the standard curve, we assayed a range of sample volumes between 50 and 120 µL. The assay measured

![Fig. 1. Composite standard curve (□) for 2-OH estrone, calculated from results (mean ± SD) of 19 RIAs carried out in triplicate over a 6-month period, and precision profile (○), representing the within-assay CVs (n = 9) for three urine samples containing a range of 2-OH estrone concentrations.](image)

<table>
<thead>
<tr>
<th>Table 1. Intra- and Interassay Variation in Quality-Control Urine Samples.</th>
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<tr>
<td>2-OH estrone conc, nmol/L</td>
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<tr>
<td>Intraassay variation (n = 9)</td>
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<td>18.0</td>
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<td>Interassay variation (n = 8)</td>
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<th>Table 2. Specificity of Antiserum Used.</th>
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<td>Steroid</td>
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<tr>
<td>2-OH estradiol</td>
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<td>Estrone</td>
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<td>2,3-Methyl esterone</td>
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<td>Estriol</td>
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*Pre- and postmenopausal women. cr., creatinine. **Total 4-OH estrogens. The following steroids were also tested and gave cross-reactivities of <0.025%: androstenedione, cholesterol, corticosterone, progesterone, testosterone, and tamoxifen.
2-OH estrone independently of volume within this range (Fig. 2). Independence of volume was also demonstrated after acid hydrolysis (data not shown).

**Stability of 2-OH estrone in urine.** Aliquot (2 mL) of first-void urine samples from two of the premenopausal controls were stored at various temperatures with and without ascorbic acid (30 g/L): −70, −20, and 4°C. 2-OH estrone was measured at weekly intervals for 6 weeks after tetrahydrofuran extraction and enzyme hydrolysis. The concentration of 2-OH estrone did not change in either set of urine samples after 41 days under any of the storage conditions. However, 2-OH estrone concentrations in samples stored in the absence of ascorbic acid were between 80.0% and 74.4% of the values in the same samples stored in the presence of ascorbic acid. We found that the combination of ascorbic acid and EDTA in the assay buffer after acid hydrolysis displaced the antiserum (B/BB 70.5% ± 2.1%) unless diluted fivefold (100% B/BB). This corresponds with the dilutions necessary to bring urinary 2-OH estrone concentrations within the range of the standard curve.

**Clinical Samples**

**Variations during the menstrual cycle.** Concentrations of 2-OH estrone were measured in 11 women during their menstrual cycles. The precise time of ovulation was not determined but was confirmed by measuring progesterone in daily saliva samples taken 7 days after commencement of menses. It was possible to give approximate time of ovulation from salivary progesterone profiles and menstrual histories (20). The concentrations of 2-OH estrone fluctuated during the cycles and showed a major preovulatory peak at midcycle (Fig. 3). Mean (±SD) concentrations in the luteal phase were higher (15.45 ± 8.8 µg/g creatinine, n = 137) than in the follicular phase (8.89 ± 5.1 µg/g creatinine, n = 131).

**Postmenopausal women.** Urinary 2-OH estrone in single samples from 14 postmenopausal controls ranged from 0.37 to 5.21 µg/g creatinine (median 0.96). In 33 postmenopausal women recently diagnosed with breast cancer, the concentrations ranged from 0.27 to 24.06 µg/g creatinine (median 1.55) (Fig. 4).

Discussion

This RIA of 2-OH estrone in urine has several advantages over published RIAs (17, 18). For example, acid was used in published RIAs to hydrolyze urinary conjugates of 2-OH estrone, but its influence on recovery of 2-OH estrone was apparently not investigated (17, 18). We compared recoveries of 2-OH estrone after acid or enzyme hydrolysis of the same urine samples and obtained values of 49.6% and 73.8%, respectively (Table 3). This difference persisted in urine samples assayed with and without the addition of 2-OH estrone. We assessed the recovery of controls with added 2-OH estrone but not with added 2-OH estrone glucuronide because we were unable to find a source to supply the latter steroid. The relatively high specificity of the antiserum precluded recovery studies with structurally related steroids such as estrone glucuronide. We therefore recommended using enzyme hydrolysis instead of acid hydrolysis.

We developed a relatively specific antiserum for 2-OH estrone and characterized its cross-reactivity against 14 steroids, including several that are structurally similar to 2-OH estrone. Only two, 2-OH estradiol and 2-OH estriol, displayed significant cross-reactivities (17.4% and 14.4%, respectively; Table 2). Concentrations of these two catechol estrogens are relatively low, even in pregnant women (17). Although it would have been useful to compare values with and without chromatography, we did not include in this RIA, because of the relatively high specificity of this antiserum for 2-OH estrone, a preliminary chromatographic purification step that is used in RIAs with less-specific antisera (17). The detection limit of this assay (100 and 240 ng/L) is better than that published for one RIA (17), and is similar to that for another (18).

Cross-reactivities of antisera are influenced by several factors that are essential components of a comprehensive validation of an immunoassay. These include determination of analytical recovery of standard analyte added to the matrix in question and independence of volume. The latter should demonstrate that the immunoassay detects the concentration of endogenous analyte in the sample without interference from other matrix constituents (24). These factors have not received sufficient attention, in our opinion, in some published RIAs of 2-OH estrone (17, 18). We demonstrated that this RIA measures 2-OH estrone in urine independently of the volume assayed, in the range 50 to 120 μL.

We confirmed previous observations that ascorbic acid stabilizes 2-OH estrone, but we also found that, in combination with EDTA in the immunoassay buffer, it could interfere with the RIA unless diluted fivefold.

We established ranges of normal values in 11 regularly menstruating women. Concentrations of 2-OH estrogens but not of 2-OH estrone alone were measured during the menstrual cycle in a previous study (25). We showed that the concentrations of 2-OH estrone fluctuate during the menstrual cycle in these women, in whom ovulation was confirmed by midcycle increases in salivary progester-


