Detection and Measurement of Urinary 2-Hydroxyestradiol 17-Sulfate, a Potential Placental Antioxidant during Pregnancy

Kaori Takanashi, Takashi Honma, Tomohiro Kashiwagi, Hideo Honjo, and Itsuo Yoshizawa

Background: Preeclampsia is associated with a quantitative imbalance between lipid peroxide and an antioxidant coproduced in the placenta. To investigate our hypothesis that 2-hydroxyestradiol 17-sulfate (2-OH-ES) is the placental antioxidant during pregnancy, we developed an assay for 2-OH-ES in urine and studied samples from women with and without preeclampsia.

Methods: The detection and measurement of 2-OH-ES in the urine of pregnant women were performed by RIA using highly specific antiserum to 2-OH-ES. To confirm the reliability of the RIA method, the same samples were analyzed by HPLC using an electrochemical detector.

Results: Urinary 2-OH-ES values obtained by RIA showed a close relationship to those obtained by HPLC ($y = 1.1x - 0.01; r = 0.96$). The urinary 2-OH-ES concentrations during the first, second, and third trimesters were $2.0 \pm 0.6$ (mean $\pm$ SE, $n = 13$), $5.3 \pm 1.3$ (n = 21), and $15.3 \pm 2.0$ $\mu$g/mg creatinine ($n = 54$), respectively, and $<0.15$ $\mu$g/mg creatinine ($n = 10$) at 2–24 h after delivery. The concentrations in preeclamptic women during the third trimester were significantly lower, $3.9 \pm 1.9$ $\mu$g/mg creatinine (mean $\pm$ SE, $n = 12$). Conclusions: RIA can be used to measure urinary 2-OH-ES during pregnancy. The increase in urinary 2-OH-ES during gestation, its decrease after delivery, and the lower values in preeclampsia are consistent with a role of 2-OH-ES as a placental antioxidant.

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Preeclampsia is a serious disease that affects many women during pregnancy, especially in the third trimester (1). This disease is now recognized pathophysiologically as an endothelial dysfunction that probably is related to the accumulation of lipid peroxides produced in the placenta (2, 3). The placenta also has opposite physiological activities, including the production of antioxidants that decompose the placental lipid peroxides (3–5). Generally, a healthy pregnancy is maintained by the quantitative balance between such oxidants and antioxidants (3–5), and the disintegration of this equilibrium might lead to preeclampsia (3–5). Until now, the kinds of substances that function as placental antioxidants have been unknown. Catechol estrogen (free form) has been considered as one of the most likely candidates, acting as an endogenous antioxidant (6). However, we suspected the effect of free catechol estrogen because of its low plasma concentration (7) and rapid metabolic clearance rate in humans (7, 8).

Previously, we reported a negative correlation between the serum concentration of estradiol 17-sulfate (ES) and those of lipid peroxides in late pregnancy (9). The results led us to speculate that ES is metabolized to its catechols, which act as lipid peroxide scavengers during pregnancy. This speculation was based on the following findings: human placental microsomes have a fairly high 2-hydroxylase activity toward ES (10), and the product 2-hydroxyestradiol 17-sulfate (2-OH-ES) has a strong inhibitory effect on lipid peroxidation (11). More recently, we reported the presence of 2-OH-ES in the blood of pregnant women and its lower concentrations in the peripheral blood of pregnant women with pregnancy-induced hypertension, compared with healthy pregnant women (12).
Therefore, whether 2-OH-ES is present in the urine of pregnant women is of particular interest.

Here, we describe our discovery of 2-OH-ES in the urine of pregnant women and its measurement during pregnancy by a previously established RIA (13). The reliability of the RIA method was confirmed by HPLC using electrochemical detection.

**Materials and Methods**

**REAGENTS**
The steroidal conjugates, ES (14), 2-OH-ES (14), 4-hydroxyestradiol 17-sulfate (4-OH-ES) (14), 2-methoxyestradiol 17-sulfate (2-OMe-ES) (14), and [6,7-3H] 2-OH-ES (1.2 × 10^12 Bq/mmol) (13) were prepared as described previously. Estriol 16-glucuronide (E3-16-G) and dextran (T-70) were obtained from Sigma. Charcoal (Norit A) was purchased from Nacalai Tesque. Dextran-coated charcoal was prepared from 50 g/L charcoal and 5 g/L dextran in assay buffer solution (15). Bovine serum γ-globulin (Cohn fraction II) and bovine serum albumin (fraction V) were purchased from Sigma. Sep-Pak C18 cartridges and Millex 4-mm HV were purchased from Waters and Millipore, respectively. All other reagents and solvents were of reagent grade.

**SUBJECT INFORMATION**
All subjects were outpatients in this study. The procedure followed was in accordance with the Helsinki Declaration of 1975 as revised in 1996. The aim of the present study was explained to each pregnant woman and puerperant, and informed consent was obtained from all women. The mean ages of the pregnant women in the first, second, and third trimesters were 30.7 years (range, 23–38 years; n = 54), respectively. The mean age of the puerperants was 28.0 years (range, 21–33 years; n = 10). No drugs were administered to any of the subjects until urine was collected.

**URINE COLLECTION**
Urine samples were obtained from healthy pregnant and preeclamptic women, and puerperants at 2–24 h after delivery. Ascorbic acid was added to the collected urine samples to a final concentration of 0.1 g/L, and urines were stored at −20 °C until analyzed. Urinary creatinine was measured by the Folin-Wo method.

**RIA**
The assay was carried out as described previously (13) except for the method of bound and free separation. To urine samples (0.2 mL, diluted with distilled water when necessary) or aqueous 2-OH-ES solution (0.2 mL; 0–100 ng) were added the following two solutions: 0.2 mL of [6,7-3H] 2-OH-ES (20 000 dpm) in 50 mmol/L Tris-HCl buffer solution (pH 7.0) containing 0.5 g/L bovine serum γ-globulin, 0.6 g/L bovine serum albumin, and 0.1 g/L ascorbic acid; and 0.6 mL of antiserum diluted 1:1000 with the same buffer solution. The mixture (1 mL) was incubated at 4 °C for 18 h, after which 0.5 mL of a suspension of dextran-coated charcoal was added. The mixture was then incubated at 4 °C for 10 min, and then centrifuged at 1500g for 10 min to remove the charcoal. The radioactivity of the duplicate supernatant fractions (2 × 0.5 mL) was measured by an Aloka LSC-1000 liquid scintillation spectrophotometer in ACS-II (Amersham) as the scintillator.

**HPLC**
Apparatus. HPLC was carried out in a model CCPS (Tosoh) equipped with an EC-8011 electrochemical detector (Tosoh) at 700 or 900 mV vs a Ag/AgCl reference electrode as described previously (16). A Mightysil RP-18GP column packed with 5-μm particles (250 × 3.0 mm i.d.; Kanto) was used as a stationary phase and maintained at 40 °C in a column heater. Data processing was performed by a Model C-R6A Chromatopac (Shimadzu).

**Separation.** The chromatographic condition for the simultaneous separation of ES, 2-OH-ES, 4-OH-ES, 2-OMe-ES, and E3-16-G were established, using various concentrations of 5 g/L NH4H2PO4 (pH 3.0) and methanol as the mobile phase and a flow rate of 0.4 mL/min.

**Calibration curve for 2-OH-ES.** The calibration curve for 2-OH-ES was obtained by injecting a known amount of 2-OH-ES and plotting the relationship between the injected amount and the peak area.

**Preparation of the 2-OH-ES-free urine.** Sulfate-free urine was prepared by the method of Heyns et al. (17). Charcoal (50 mg) was added to 1 mL of a pooled urine sample, and the mixture was stirred vigorously for 30 min at room temperature. The mixture was centrifuged at 1500g for 10 min to remove the charcoal, and the supernatant was filtered through a membrane filter to give the 2-OH-ES-free urine.

**Recovery of 2-OH-ES.** A known amount of 2-OH-ES was added to the 2-OH-ES-free urine, and the mixtures were analyzed by HPLC.

**Comparison of urinary 2-OH-ES concentrations measured by RIA and HPLC**
Urine samples collected from pregnant women and from puerperants were divided into two fractions, and 1 mL of each fraction was analyzed by RIA and HPLC as described.

**Urinary 2-OH-ES**
Measurement of urinary 2-OH-ES was carried out as follows: A 1-mL aliquot of each urine sample was passed through a Sep-Pak C18 cartridge, followed by washing with 2 mL of distilled water. The steroid-containing fraction was obtained by elution with methanol (4 mL), and the eluates were evaporated under a nitrogen stream at 40 °C to give the residue, which was dissolved in 100
µL of methanol containing 0.1 g/L ascorbic acid. After being passed through a Millex 4-mm HV filter, the solution was subjected to HPLC.

**Results**

Urinary 2-OH-ES was measured by RIA with a slight modification of the previous method (13). In constructing the calibration curve, we used 1000-fold diluted antiserum and plotted the percentage of labeled 2-OH-ES bound against various amounts of 2-OH-ES. The calibration curve obtained was linear for 0.05–100 ng of 2-OH-ES. Urinary 2-OH-ES concentrations measured by this method were expressed as µg/mg creatinine.

In the present report, the HPLC detection and assay method for urinary 2-OH-ES was established to confirm the RIA method. The chromatographic conditions for quantitative analysis of urinary estrogen conjugates were established using such authentic conjugates as ES, its potential ring-A metabolites (2-OH-ES, 4-OH-ES, and 2-OMe-ES), and E3-16-G (18), whose urinary excretion rate is known to increase during pregnancy. When a 60:40 (by volume) mixture of 5 g/L NH₄H₂PO₄ (pH 3.0) and methanol was used as the mobile phase, the above authentic steroids were separated satisfactorily, as shown in Fig. 1.

The conditions for sensitive detection of 2-OH-ES were established by controlling the detector voltage of the electrochemical detector. As described previously (16), catechol- or guaiacol-type conjugates could be detected with adequate sensitivity even in the presence of phenol-type conjugates by controlling the detector voltage. Fig. 1 shows the results obtained, where solid and dotted lines represent the chromatograms detected at 700 and 900 mV, respectively. There were almost no differences in the peak heights of the catechol- or guaiacol-type conjugates detected at 700 or 900 mV. In sharp contrast, when the voltage was lowered from 900 to 700 mV, the peak heights of phenol-type conjugates were substantially reduced or not detectable. These results indicated that sensitive analysis of 2-OH-ES could be accomplished by decreasing or eliminating the influence of the large amounts of the phenol-type conjugates present in pregnancy urine.

A representative HPLC chromatogram of the estrogen-containing fraction from pregnancy urine, which was obtained at the detection voltage of 700 mV, is shown in Fig. 2. Peak assignment in the chromatogram was achieved by the addition of authentic conjugates to the sample. The lowering of the voltage from 900 to 700 mV reduced the size of the E3-16-G peak substantially, allowing the discrimination of small peaks of catechol- or guaiacol-type conjugates.

The calibration curve was constructed by plotting the peak area against the amount of 2-OH-ES injected, and satisfactory linearity was observed for 2-OH-ES concentrations of 2–200 ng \( (y = 1.14x + 0.014; r = 0.998) \). To confirm the validity of the method for the determination of urinary 2-OH-ES, a known amount of authentic 2-OH-ES was added to the sulfate-free urine, and the conjugate recovered through the whole clean-up procedure was determined. It is evident from the data in Table 1 that 2-OH-ES was recovered to a satisfactory extent.

Once the analytical method of urinary 2-OH-ES by HPLC was satisfactorily established, the reliability of the RIA method was verified. The urine sample was divided into two parts, each of which was analyzed by RIA and HPLC. As indicated in Fig. 3, the urinary 2-OH-ES values were consistent between the two methods, confirming the validity of the RIA method.
obtained by RIA showed a close relationship to those obtained by HPLC; the equation in the regression line was: \( y = 1.10x - 0.0143 \) (\( r = 0.958; n = 35; P < 0.01 \)). The RIA method thus was demonstrated to be useful. Because of its simplicity, ease of use, and capacity for handling numerous samples, the measurement of 2-OH-ES in the urine from pregnant women or puerperants was carried out by the RIA method.

The urinary 2-OH-ES concentrations of pregnant women during gestation are shown in Fig. 4, where circles (○) and triangles (△) indicate healthy pregnant women and preeclamptic women, respectively. The urinary 2-OH-ES concentrations of healthy pregnant women increased as the gestation progressed; the concentrations in the first, second, and third trimesters were 2.0 ± 0.6 (mean ± SE; n = 13), 5.3 ± 1.3 (n = 21), and 15.3 ± 2.0 (n = 54) \( \mu \text{g}/\text{mg creatinine}, \) respectively. The concentrations during the third trimester were seven- and threefold higher than those during the first and second trimester, respectively. The concentration in the preeclampsia cases was 3.9 ± 1.9 \( \mu \text{g}/\text{mg creatinine} \) (mean ± SE; n = 12), which is approximately one-fourth the value in healthy cases in the same stage. The differences in urinary 2-OH-ES concentrations between the first and second trimesters, and the second and third trimesters were significant, at \( P < 0.05 \) and \( P < 0.01 \), respectively. The difference in the urinary concentrations between healthy pregnant women and preeclamptic women during the third trimester was also significant, at \( P < 0.01 \). The 2-OH-ES concentrations at 2–24 h after delivery decreased to <0.15 \( \mu \text{g}/\text{mg creatinine} \) (n = 10); this value was irrelevant to the clinical history of pregnant women with or without preeclampsia.

**Discussion**

In the present study, it became evident that 2-OH-ES is excreted into the urine of healthy pregnant women in fairly high concentrations. As far as we know, this is the first report describing the detection of this estrogen sulfate in human urine. In addition, we demonstrated that urinary 2-OH-ES increases as gestation progressed, which is considered quite natural because of similar evidence that urinary ES increases with the progress of gestation (19), and because of the high 2-hydroxylase activity toward ES by human placental microsomes (10). Our recent report (12) dealing with the existence of this sulfate in the serum of pregnant women also supports the present results.

As reported previously, the antiserum used in this

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**Table 1. Recoveries of 2-OH-ES added to the sulfate-free pregnancy urine.**

<table>
<thead>
<tr>
<th>Added</th>
<th>Detected*</th>
<th>Recovery,*%</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>24.4 ± 0.8</td>
<td>97.5 ± 2.5</td>
<td>2.6</td>
</tr>
<tr>
<td>100</td>
<td>97.6 ± 2.4</td>
<td>97.6 ± 2.4</td>
<td>2.5</td>
</tr>
<tr>
<td>200</td>
<td>190.2 ± 1.6</td>
<td>95.1 ± 0.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*Values are means ± SD; n = 3.
assay was highly specific for 2-OH-ES, and its cross-reactivities with similar estrogen conjugates were extremely low (13). For example, the cross-reactivities of such similar conjugates as ES, 2-OMe-ES, and 4-OH-ES, were 0.1%, 0.03%, and 0.01%, respectively. Even E3-16-G (18), excreted in large quantity into the urine during pregnancy, showed a cross-reactivity <0.01%. However, there still remains the possibility of the presence of unknown urinary substance(s) with high cross-reactivity to the antibody. To ensure that the above RIA is applicable to the measurement of urinary 2-OH-ES, we developed the HPLC method for the same urine samples.

As shown in Fig. 3, urinary 2-OH-ES values obtained by RIA exhibited a close correlation to those obtained by HPLC. The RIA established thus is reliable and applicable to the urine samples. The measurement of urinary 2-OH-ES was carried out by RIA because it was superior to HPLC in its ability to deal with a large number of samples.

In Fig. 4, urinary 2-OH-ES is shown to increase as gestation progresses, especially in the third trimester. Although the concentrations seem to be uneven among individuals, the urinary 2-OH-ES concentration (15.3 ± 2.0 μg/mg creatinine) in the third trimester is significantly higher than in the first two trimesters, and is ~370-fold higher than the urinary ES (41.4 ± 4.7 ng/mg creatinine) at the same stage, as reported previously (19). The big difference between the urinary concentrations of ES and 2-OH-ES implies the conversion of ES to 2-OH-ES during pregnancy, especially in the last stages of the pregnancy. This can be explained by the presence of high 2-hydroxylation activity toward ES in the placental microsomes (10). These results suggest the physiological requirement of this aromatic hydroxylation during pregnancy.

As candidate substances for the placental antioxidants, there have been several reports nominating superoxide dismutase (20–22), catalase (21, 22), glutathione peroxidase (21, 22), or catechol estrogens (6). Of these, superoxide dismutase, catalase, and glutathione peroxidase are not now recognized as participating actively in the maintenance of healthy pregnancy because there seem to be no increasing tendencies in their production with the progression of gestation (22). Catechol estrogens such as 2-hydroxyestrone or 2-hydroxyestradiol have recently attracted considerable attention as the most possible candidates, especially because of their strong antioxidant effect (23). However, it seems that some inexplicable problems remain in the assignment of catechol estrogens as placental antioxidants. Plasma circulating catechol estrogens are known to be rapidly metabolized by erythrocytic catechol O-methyltransferase to the corresponding guaiacol estrogens, such as 2-methoxyestrone or 2-methoxyestradiol (7, 8). The plasma concentration of catechol estrogens thus is extremely low (7). Although guaiacol estrogens evidently have a strong antioxidant effect (11), we cannot consider them as placental antioxidants because they bind strongly or irreversibly to plasma testosterone-estradiol binding globulin (24).

In contrast to free-formed catechol estrogens, the C17-sulfoconjugated estrogen (2-OH-ES) has some properties that may make it advantageous as a placental antioxidant: (a) the 2-hydroxylase activity of placental microsomes is higher toward ES than estradiol (10); (b) the plasma metabolic clearance rate of 2-OH-ES is lower than that of 2-OH-E in rats (25); (c) 2-OH-ES has a strong antagonistic effect against lipid peroxidation (11); and (d) 2-OH-ES concentrations are significantly higher in umbilical arteries than in the maternal peripheral vein (12). In addition, the two results observed in the present study—the rapid decrease in urinary 2-OH-ES after delivery, and the lower urinary 2-OH-ES concentrations in preeclampsia compared with healthy pregnancy—may support our hypothesis.

In conclusion, 2-OH-ES may be clinically important in the field of obstetrics. In this report, urinary 2-OH-ES was measured directly by RIA without hydrolysis, the methodology was shown to be simple and easy, and the reliability of the method was supported by comparison with another method (HPLC). Further detailed studies, such as a follow-up measurement of urinary 2-OH-ES in individual subjects, or an investigation of the effect of medical treatment on urinary 2-OH-ES in preeclamptic women, may provide additional evidence of its usefulness as a marker for the diagnosis of preeclampsia. The RIA established here may become a powerful tool in the development of this study.

This work was supported in part by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education (1996, K. Takanashi) and the Akiyama Research Grant from The Akiyama Foundation (1998, K. Takanashi).

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