Estrone Sulfate Concentrations in Plasma of Normal Individuals, Postmenopausal Women with Breast Cancer, and Men with Cirrhosis

Agnès Rémy-Martin, Odile Prost, Monique Nicollier, Josette Burnod, and Gérard L. Adessi

Estrone sulfate is quantitatively the most important estrogen in plasma. A method for its determination in human plasma is described, and the precision, accuracy, sensitivity, and specificity are defined. Free steroids were extracted from plasma with diethyl ether and steroid sulfates were isolated with use of Vitlos' reagent (methylene blue in dilute H2SO4/Na2SO4 solution). After enzymic hydrolysis, estrone was isolated by chromatography on Celite and measured by radioimmunoassay. The mean concentrations (nmol/L ± 1 SD) of estrone sulfate were 2.51 ± 0.90 nmol/L for plasma from 13 women in follicular phase, 5.33 ± 1.55 for 17 women in luteal phase, 0.89 ± 0.60 for 44 postmenopausal women, and 0.96 ± 0.43 for 24 postmenopausal women with breast cancer.

Results for postmenopausal women with or without breast cancer did not differ significantly. For 13 normal men, estrone sulfate concentrations were 2.62 ± 0.79 nmol/L, and for a group of 19 cirrhotic men the mean value was 1.43 ± 0.95 nmol/L, significantly lower than normal.

Additional Keyphrases: estrogens • hormones • radioimmunoassay • chromatography, column • reference intervals

Estrone sulfate (E1S), present in plasma in a higher concentration than either unconjugated estrone or estradiol-17β in nonpregnant women and normal men (1), appears to originate almost entirely from a conjugation of estrone and converted estradiol-17β in nonpregnant tissues (2). Moreover, estrone sulfate is only slowly cleared from plasma (3), thus its concentration does not fluctuate markedly during the day (4). Interest in a plausible physiological role of estrone sulfate in mammalian tissue increased greatly after hydrolysis of this component was demonstrated in a responsive estrogen tissue such as human endometrium (5), and its conversion into estradiol-17β in sheep myometrium (6), sheep fetal pituitary (7), and guinea pig uterus (8). More recently, Vignon et al. (9) concluded that estrone sulfate can act as an estrogen precursor in human breast cancers that contain sulfatase, at least in vitro.

Techniques reported for measuring estrone sulfate in plasma involve its deconjugation by enzymic hydrolysis (10–12) or solvolysis (1, 4, 13, 14) before separation and radioimmunoassay (1, 10–14) or radio-competition assay (4) of the liberated unconjugated estrone. Direct radioimmunoassay of the intact conjugate has been hampered by the lack of specific antisera (3, 15). Wright et al. (3) used an antiserum raised against estrone glucosiduronic-bovine thyroglobulin. More recently, a specific radioimmunoassay for estrone sulfate in plasma was reported (16).

The technique used in the present study includes the specific extraction of plasma sulfates by use of the Vitlos reagent (17), enzymic hydrolysis, Celite column chromatography, and radioimmunoassay of the isolated unconjugated estrone. We applied this technique to samples obtained during the normal menstrual cycle in women, from postmenopausal women, and from men. We also measured the concentrations of estrone sulfate in plasma from postmenopausal women with breast cancer and from men with hepatic cirrhosis, a disorder potentially associated with a defect in hepatic sulfurylation (14).

Materials and Methods

Reagents

[6,7-3H]Estrone-3-sulfate (53 kCi/mol) was obtained from New England Nuclear, Paris, France. Estrone-3-sulfate (E1S), sodium salt, was purchased from Sigma Chemical Co., St. Louis, MO 63178. Solutions were prepared in ethanol and stored below 0 °C. Before use, any unconjugated estrone produced by spontaneous hydrolysis was extracted by diethyl ether-water partitioning. All solvents (E. Merck, Darmstadt, G.R.) were of analytical grade. Vitlos reagent was prepared according to the method of Goertz et al. (17): 250 mg of methylene blue (cat. no. MB-1, Sigma Chemical Co.) was dissolved in 300 mL of distilled water, and 50 g of Na2SO4 (Merck) and 10 mL of sulfuric acid (no. 714, Merck) were added. Redistilled water was added to give a final volume of 1 L. The filtered solution was stored protected from light, at 4 °C.

Helix pomatia juice [106 (E3.1.6.1) and 106 Fishman units of β-glucuronidase (3.2.1.31) per milliliter] was purchased from Industrie Biologique Française (Pharmindustrie, Gennevilliers, France). We purified it according to the method of Jarrige (18) and kept it at 4 °C for two weeks without loss of activity. Immediately before assay, the purified juice was diluted 10-fold with acetate buffer (0.1 mol/L, pH 6) and 3 g of moist Amberlite XAD 2 (Serva, Heidelberg, F.R.G.) was added to 10 mL of the diluted juice. This mixture was agitated on a stirring wheel for 15 min at 4 °C, then filtered through filter paper ("Enzymatic preparation").

Celite (Chromatolithe A; BioMérieux, Lyon, France) was used according to the manufacturer's instructions.

Estrone, estradiol-17β, progesterone, and folitropin were measured with commercial RIA kits from BioMérieux.

For liquid scintillation spectrometry we used 4 mL of PicoFluor 15 (Packard Instrument Co., Rungis, France). Radioactivity was measured in a Kontron Model MR 300 liquid-scintillation spectrometer with a counting efficiency of 50% for tritium (external standardization method). Sufficient counts were accumulated for all samples to give counting errors of <2%.

Glassware was washed with a detergent (Extran; Merck), then with chromosulfuric acid, and rinsed with tap water and redistilled water. The glass tubes used for the extraction were deactivated with a mixture of dimethyldichlorosilane/chloroform (1:10 by vol) then thoroughly rinsed with methanol and redistilled water before use.
Procedure

Blood was sampled, with EDTA as anticoagulant, by venipuncture between 0800 and 1000 hours. The subjects had fasted for 12 h. The plasma was promptly frozen and stored at −20 °C in several assay tubes until processing.

To 0.5 or 1 mL of plasma, add 7000 dpm of [3H]E2S in 20 μL of water, to allow correction for manipulative losses. When 0.5 mL is used, adjust the volume to 1 mL with ammoniacal water (NH₄OH/H₂O, 1/1000, by vol). Mix the contents of the tubes thoroughly and allow to settle at room temperature for 30 min. Then extract with 5 mL of diethyl ether. Discard the ether extract, and briefly heat the aqueous phases at 40 °C under a gentle stream of nitrogen before adding 2 mL of ammoniacal water and 1 mL of Vlitos reagent. Extract the methylene blue—steroid sulfate complex with chloroform (once with 8 mL and twice with 4 mL) by mechanical shaking for 10 min, centrifuge at 1000 × g for 10 min, and decant the organic phases. Combine the organic phases, wash with 4 mL of distilled water, and evaporate. Add 1.5 mL of distilled water to the residue, briefly sonicate (Bransonics; Bioblock; Ilikirkich, France), and incubate for 10 min at 55 °C to ensure complete solubilization. Hydrolyze the extracted steroids enzymatically by incubating with 0.5 mL of the "Enzymic preparation" for 1 h at 55 °C. After cooling, extract the unconjugated steroids with 10 mL of diethyl ether. Discard the aqueous phases after freezing in an ice/NaCl bath, and evaporate the organic phases under nitrogen at 40 °C in clean tubes. Transfer the residues to the Celite column by use of two 1-mL portions of isooctane (allow the first portion wash to stand in the tube for 15 min at room temperature), and the first transfer pass into the column before adding the second. Add 4 mL of isooctane to the column, then 5 mL of isooctane/ethyl acetate (94/6 by vol). Elute the estrone into clean tubes with 6 mL of isooctane/ethyl acetate (80/20 by vol), then evaporate the solvent under nitrogen at 60 °C. Add 0.5 or 1 mL of phosphate buffer (0.1 mol/L, pH 7.4) vortex-mix, and let the tubes stand at room temperature for 30 min before performing the RIA. Determine the analytical recovery of estrone by counting the radioactivity of a 0.2- or 0.5-mL aliquot of this phase.

To determine estrone by RIA, we used a commercial kit from BioMérieux and duplicate 0.1-mL aliquots of the extracted samples. We prepared standard curves, using 0, 23.2, 46.3, 92.6, 185.2, 370.4, 740.7, and 1481.4 fmol purified estrone. To each assay tube (standard or sample), we added 0.1 mL of phosphate buffer (10 mmol/L, pH 7.4) containing estrone antiserum and 0.1 mL of phosphate containing approximately 12 000 dpm of [6,7-3H]estrone, mixed well, and incubated them at 4 °C overnight. Then we added 0.5 mL of dextran-coated charcoal suspension to each tube and mixed thoroughly. The tubes were allowed to stand for 10 min at 4 °C and centrifuged for 10 min at 2800 rpm, after which the supernates were decanted into counting vials and their radioactivity was counted. The amounts of estrone in the sample tubes were calculated from a logit-log plot. The results were corrected for recovery and expressed as nanomoles of estrone sulfate per liter.

Results

Analytical Criteria

Standard curves: Typical standard curves (n = 10) obtained over a continuous period of 10 weeks are shown in Figure 1. The standard curve was useful over the range 23.2–740.7 fmol, with 50% displacement at 185 fmol. Precision: Within-run coefficients of variation were estimated by assaying, in the same run, samples from three different plasma pools. Between-run precision was assessed by the differences between duplicate determinations in different runs. Standard deviations were calculated according to Snedecor (19). The results are shown in Table 1.

Recovery: The mean analytical recovery of the internal standard [6,7-3H]E2S added to the plasma before processing was 33.8% (SD 8.9%) over 259 assays. For these experiments we used a pool of plasma from men, to which we added four different amounts of estrone sulfate (Table 2). Each value was corrected for methodological losses. In each case, the amount recovered did not differ significantly from the amount added.

Sensitivity: Distilled water was used to determine blank values, because charcoal treatment cannot completely eliminate estrone sulfate from plasma (8). The ratios B/B₀ were always higher than those obtained for the lowest point of the standard curve (23.2 fmol per tube). When we assayed samples of plasma free of estrone sulfate—i.e., horse plasma—the values for estrone sulfate were consistently indistinguishable from blank values.

Reproducibility: Plasma (1 mL per assay) with a low concentration of E₂S was processed (n = 15) in the same run. The mean value obtained was 494 pmol/L, with a standard deviation of 53 pmol/L.

Specificity: When we added 25.6 nmol of estrone β-glucuronide per liter, in 20 μL of ammoniacal water, to plasma and assayed with the proposed method, we found less than 1%. When smaller quantities of estrone glucuro-
Table 2. Analytical Recovery of Estrone Sulfate Added to Plasma

<table>
<thead>
<tr>
<th>Added, nmol/L*</th>
<th>Accounted for, %b</th>
<th>SEM, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.89</td>
<td>109.0</td>
<td>8.3</td>
</tr>
<tr>
<td>3.79</td>
<td>102.1</td>
<td>3.1</td>
</tr>
<tr>
<td>5.68</td>
<td>100.9</td>
<td>5.2</td>
</tr>
<tr>
<td>7.58</td>
<td>100.7</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*Amount before adding sulfate: 2.15 (SD 0.23) nmol/L. b Mean of triplicate determinations.

We found no estrone was detected in the plasma of normal menopausal women (ND).

Specificity of the method was achieved by isolating steroid sulfates by use of the Vlitos reagent, by using Celite column chromatography to isolate estrone, and because of the specificity of the immunoreagent (according to the manufacturer, BioMérieux).

Clinical Results

Women. We measured the concentrations of estrone sulfate, estradiol-17β, and progesterone in the plasma of 94 women (Table 3). The hormonal status was established by the patient's medical background; the basal body-temperature chart for menstrual cyclic women or follicular concentrations in plasma for postmenopausal women (39.5 ± 10.7 int. units/L) and postmenopausal women (41.2 ± 21.0 int. units/L) were collected. Estrone sulfate concentrations were determined.

Added, estrone for postmenopausal women with breast cancer, plasma was collected before the women underwent mastectomy. Mammary cancers were confirmed by pathological examination. Estrone sulfate concentrations exceeded those of unconfounded estradiol by 7.8 times in the follicular and luteal phases, by 9.1 times in postmenopausal women, and by 12.0 times in postmenopausal women with breast cancer. Mean values for the luteal phase were significantly (p < 0.001) higher than those for the follicular phase. There was no significant difference in estrone sulfate concentrations between postmenopausal women with and without breast cancer. The values for postmenopausal women were significantly lower than those for cyclic menstrual women (follicular phase p < 0.001; luteal phase p < 0.001).

Men. Estrone sulfate was measured in plasma samples from 13 normal men (Table 3). The mean value is significantly lower than that obtained for normal women (luteal phase) (p < 0.001) but higher than that obtained for postmenopausal women (p < 0.001). The 19 men with liver disease (cirrhosis established by laparoscopy) had a mean value lower than that for normal men (p < 0.01). The mean unconjugated estrone concentration in these men with liver disease, 0.42 (SD 0.30) nmol/L, is higher than that reported for normal men by others (4, 11).

Discussion

Estrone and other unconjugated estrogens are almost completely removed (>95%) by pre-extraction with diethyl ether. Estrone glucosiduronate, like all glucosiduronates, is not extracted by the Vlitos reagent (17); thus the fraction submitted to hydrolysis contained only steroid sulfates. The antisperm specificity (cross reaction with estradiol-17β was 2.9%, with estradiol 0.04%, with neutral steroids 0.001%) may allow valid radioimmunoassay without further purification of the hydrolyzate. High blank values were found by this procedure; low values were obtained only after isolation of estrone by Celite column chromatography.

A strict comparison of the values for estrone sulfate in plasma reported by different workers is difficult because of the differences in methodology (Table 3).

Loriaux et al. (1) and Franz et al. (14) were unable to demonstrate a significant increase in circulating estrone sulfate in the luteal phase of the cycle as compared with the early follicular phase. In agreement with Wright et al. (3) and Núñez et al. (13), we found that estrone sulfate increases significantly in the luteal phase as compared with the follicular phase.

In postmenopausal women, the values for estrone sulfate reported here are in agreement with those of other authors (10, 12, 14). Estrone sulfate is quantitatively the most important circulating estrogen. In postmenopausal women with breast cancer, estrone sulfate concentrations in plasma have the same order of magnitude. Breast tumors contain sulfatase activity (20) and can convert estrone sulfate into estradiol-17β (21). Consequently, estrone sulfate provides a continuous supply of estrogens to hormone-responsive tumors. Vignon et al. (8) provided direct evidence that estrogen sulfates can behave as biologically active estrogens in breast cancer cells maintained in culture. Thus it would appear that plasma estrone sulfate should be taken into consideration in determining the estrogen balance in women with breast cancer.

In the group of cirrhotics, we found, as did Franz et al. (14), that the values for E,S were significantly lower than the values for normal men. These authors concluded that a defect in the sulfurylation of estrone, probably at the hepatic level, is the most likely reason for the lower E,S concentrations in hepatic cirrhosis. However, in normal men, Wright et al. (3) have shown that there is a net uptake of estrone sulfate in the splanchnic area, which does not then release

Table 3. Plasma Estrone Sulfate Concentrations

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age, *years</th>
<th>Weight, *kg</th>
<th>Estradiol*</th>
<th>Progesterone*</th>
<th>Estrone sulfate**</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>nmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular phase</td>
<td>13</td>
<td>32.8 (5.8)</td>
<td>56.1 (6.0)</td>
<td>0.32 (0.12)</td>
<td>2.1 (0.8)</td>
<td>2.51 (0.90)</td>
<td>Present data</td>
</tr>
<tr>
<td>Luteal phase</td>
<td>17</td>
<td>28.5 (4.4)</td>
<td>56.1 (6.2)</td>
<td>0.73 (0.27)</td>
<td>48.5 (27.5)</td>
<td>5.33 (1.55)</td>
<td>Present data</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>44</td>
<td>67.5 (10.8)</td>
<td>59.9 (12.7)</td>
<td>0.10 (0.05)</td>
<td>1.7 (0.9)</td>
<td>0.89 (0.60)</td>
<td>Present data</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>24</td>
<td>65.7 (12.3)</td>
<td>ND</td>
<td>0.08 (0.03)</td>
<td>2.9 (1.7)</td>
<td>0.96 (0.43)</td>
<td>Present data</td>
</tr>
<tr>
<td>Men</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal men</td>
<td>13</td>
<td>35.9 (11.2)</td>
<td></td>
<td>2.62 (0.79)</td>
<td></td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td>Cirrhotic men</td>
<td>19</td>
<td>50.8 (14.7)</td>
<td></td>
<td>1.43 (0.95)</td>
<td></td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td>Normal men</td>
<td>30</td>
<td>ND</td>
<td></td>
<td>1.22 (0.41)</td>
<td></td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td>Normal men</td>
<td>19</td>
<td>ND</td>
<td></td>
<td>1.31 (0.07)</td>
<td></td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td>Normal men</td>
<td>6</td>
<td>ND</td>
<td></td>
<td>1.92 (0.80)</td>
<td></td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td>Normal men</td>
<td>13</td>
<td>ND</td>
<td></td>
<td>2.48 (0.17)</td>
<td></td>
<td></td>
<td>(7)</td>
</tr>
</tbody>
</table>

*Mean (and SD). b Duplicate determinations. c Plasma collected on day six of the menstrual cycle. d Plasma collected 6–7 days after the basal body temperature had increased. ND, not determined.
significant amounts of estrone sulfate into the blood. Another possible explanation for the decrease in estrone sulfate and the increase in unconjugated estrone is that estrone sulfate is more rapidly metabolized than unconjugated estrogens. Estrogen sulfates are tightly bound to human serum albumin (22). The lower concentrations of this protein circulating in men with cirrhosis may increase the rate of metabolism of estrone sulfate into unconjugated estrone.

This work was supported by a grant from the Institut National de la Santé et de la Recherche Médicale N° 80 20 20 and from the Université de Franche-Comté (Besançon).

References