Rapid, convenient radioimmunoassay of estrone sulfate

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We developed a specific, simple, and rapid RIA for the direct quantification of estrone sulfate (E1S) and established its performance characteristics. The assay has a dynamic range of 0.05–90 μg/L with a detection limit of 0.009 μg/L. Intraassay CVs were 9.2%, 4.5%, and 4.6% at 0.35, 9.0, and 60 μg/L, respectively. Interassay CVs were 8.8%, 5.1%, and 5.5% at 0.076, 0.5, and 12 μg/L, respectively. Linearity of dilution studies showed values of 80–105% of expected, and recovery of E1S added to serum samples ranged from 82% to 102%. Cross-reactivities with structurally related estrogens were <5%. When compared with a conventional assay (involving hydrolysis of E1S and indirect measurement of estrone), the present RIA showed excellent correlation (r = 0.99, slope = 1.54, S_yx = 2.14, n = 71). Mean E1S concentrations measured with this RIA for normal men (n = 20) and women in follicular (n = 20) and luteal (n = 25) phases of their menstrual cycle were 0.96, 0.96, and 1.74 μg/L, respectively. Mean E1S concentrations for oral contraceptive users (n = 20) and postmenopausal women without hormone replacement therapy (n = 21) or on hormone replacement therapy (n = 22) were 0.74, 0.13, and 2.56 μg/L, respectively. Serum concentrations of E1S in pregnant women in their first (n = 14), second (n = 17), and third (n = 15) trimesters were 20, 66, and 105 μg/L, respectively. Availability of this simple RIA should provide a useful tool for the assessment of estrogen status in women.

Estrone sulfate (E1S)5 is quantitatively the most important circulating estrogen in a woman [1, 2]. Formation of E1S occurs in a variety of tissues in the body but primarily in the liver. A number of tissues in the body contain sulfatases that can transform the biologically inactive E1S, by hydrolysis, to estrone (E1). The latter estrogen can then be converted to the potent estrogen, estradiol (E2), through action of the enzyme, 17β-hydroxysteroid dehydrogenase. Thus, it appears that the body keeps a reserve of estrogen in an inactive form, and when more of the active estrogen is needed, it becomes readily available.

Conventionally, E1 and E2 have been used as the markers of estrogenicity, in spite of their limitations, such as low serum concentrations and diurnal variations, which can be misleading for clinicians in patient management, depending on the time of collection of serum samples. E1S has a clear advantage over the existing markers because: (a) serum concentrations of E1S are 10–20-fold higher than E1 or E2; therefore, reliable determination of E1S concentrations is possible and would minimize the interassay variation; (b) E1S has a slow metabolic clearance rate, which would maintain the constant serum concentration with no diurnal variation; (c) E1S does not bind to sex hormone-binding globulin, whereas E2 does; as a result, the measurement of E2 with most commercially available immunoassays reflects large interlaboratory variability because of differences in affinities and specificities of antibodies toward the sex hormone-binding globulin-bound form and the free form of E2.

Until now, the limited utility of E1S as an estrogenicity marker could be attributed to the lack of availability of a simple and highly specific assay for E1S. The conventional assays for the measurement of E1S are either cumbersome

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5 Nonstandard abbreviations: E1S, estrone sulfate; E1, estrone; E2, estradiol; BSA, bovine serum albumin; CMO, (O-carboxymethyl)-oxime; and HRT, hormone replacement therapy.
and time consuming, involving hydrolysis of E₁S to E₁ followed by the extraction and indirect determination of E₁S via measurement of E₁ [3], or lack antibody specificity. For example, two earlier reports described the measurement of E₁S using antibodies that were not raised specifically against E₁S but against its nonsulfated derivatives such as estrone glucuronide and estrone 3-methylphosphorothionate [4–6]. Both reports describe the use of ³H-labeled E₁S, which has the obvious limitation of lower sensitivity. Another report used a detection system based on reactions of free radicals; its dynamic range was narrow: 5–1000 ng/L [7].

In this report, we: (a) describe the development and performance characteristics of a simple, rapid, and highly specific RIA for the direct measurement of E₁S in serum or plasma; (b) compare the new assay with an established conventional method requiring hydrolysis, extraction, and indirect determination of E₁S through measurement of E₁; and (c) establish reference ranges for men and women to expand the clinical utility of this assay.

Materials and Methods

SUBJECTS
Male volunteers were apparently healthy and not on any medication. Normally cycling women in this study were arbitrarily divided into luteal and follicular phase on the basis of cycle length and endocrine status and did not exhibit any symptoms of hyperandrogenism. A group of women on oral contraceptives was also investigated. Postmenopausal women were those who had not had a regular menstrual cycle for at least 9 to 12 months and who were in their late 40s to early 50s. Cross-sectional samples were obtained from normal singleton pregnant women at the first, second, and third trimesters.

MATERIALS
E₁S and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. All other chemicals were purchased from Aldrich Chemical Co. unless stated otherwise.

Radioactivity was measured in the Packard Multichannel RIA System γ Counter (Model B 5424).

PREPARATION OF ASSAY COMPONENTS

Synthesis of estrone-3-sulfate-6-(O-carboxymethyl)-oxime (CMO). Estrone-6-CMO (20 mg, 0.056 mmol) and sulfur trioxide–triethylamine complex (30 mg, 0.215 mmol) in dry pyridine (0.3 mL) were stirred at room temperature for 16 h. The solvent was removed under vacuum. The crude product was purified by preparative thin-layer chromatography on silica gel (Analtech; 250-μm particles) by using chloroform:methanol:ammonium hydroxide (15:5:1 by vol) as a solvent system. The yield of diammonium salt of estrone-3-sulfate-6-CMO was 26.5 mg.

Synthesis of estrone-3-sulfate-6-CMO:BSA conjugate. Estrone-3-sulfate-6-CMO diammonium salt (43.2 mg, 0.0916 μmol) in dimethylformamide (3.0 mL) was added to 1,4-dioxane (2.0 mL). The mixture was cooled to 4 °C, followed by the addition of tri-n-butylamine (23.2 μL). The mixture was stirred for 10 min, followed by the addition of isobutyl chloroformate (17.4 μL). The resulting solution was added dropwise to the solution containing water (6 mL), dioxane (2 mL), and BSA (98.5 mg, 1.53 μmol) at 2–4 °C. The pH was maintained between 8.0 and 8.5 during the addition. The turbid reaction mixture was stirred at 4 °C for 20 h. The mixture was dialyzed against 50 mmol/L sodium bicarbonate (pH 8.5) with two changes, followed by dialysis against water. The dialysate was lyophilized to give E₁S-6-CMO:BSA conjugate (65 mg). Fig. 1 illustrates the synthesis of this immunogen.

Synthesis of estrone-3-sulfate-6-CMO-histamine. Estrone-3-sulfate-6-CMO (5.6 mg), diisopropylethylamine (4 μL), histamine (2 mg, 0.018 μmol), and diethylphosphoryl cyanide (5 μL) in dry dimethylformamide (160 μL) were stirred at room temperature for 24 h under argon. The solvent was removed under vacuum, and the residue was purified on preparative thin-layer chromatography by using chloroform:methanol:ammonium hydroxide (15:5:1 by vol) as a solvent system. Estrone-3-sulfate-6-CMO-histamine was obtained as an amorphous white solid (4.3 mg; Fig. 1) as a precursor for ¹²⁵I-labeled tracer.

¹²⁵I-labeled estrone sulfate tracer. Estrone-3-sulfate-6-CMO-histamine was iodinated with Na¹²⁵I by using Chloramine-T [8] in phosphate-buffered saline. The radioactive compound was then purified by reversed-phase chromatography on an octadecylsilyle column by using methanol:water containing triethylamine, 1 mL/L, as an elution system. The purified compound (~1.4 mCi/μg) was stored undiluted at −20 °C.

Generation of antiserum. Two rabbits were immunized with estrone-3-sulfate:BSA conjugate by following the protocol described previously [9]. Briefly, the primary injection (2 mg) of the conjugate in isotonic saline was mixed with an equal volume of Freund’s complete adjuvant at a final dilution of 1 g/L. It was divided in four equal portions and injected intramuscularly into each thigh and below each shoulder blade. Three booster shots were given (0.5 mg) on days 7, 14, 21, and every 30 days thereafter (50 μg/rabbit). Serum was collected 14 days after the third booster and every 30 days thereafter. These antisera were tested for their ability to bind to E₁S tracer. Antiserum from one rabbit displayed high titer and was able to displace the iodinated antigen tracer with increasing concentrations of unlabeled analyte.

RIA FOR DIRECT MEASUREMENT OF ESTRONE SULFATE IN SERUM OR PLASMA
Calibrators, controls, or unknown patient samples (100 μL) were added to the polypropylene tubes, followed by E₁S ¹²⁵I-labeled tracer (100 μL) and E₁S antiserum (100 μL). The tubes were incubated at room temperature on a
shaker for 3 h, followed by the addition of the precipitating reagent, goat anti-rabbit IgG (1 mL). The samples were allowed to stand for 10 min, followed by centrifugation for 15 min at 1500g. The supernate was then decanted, and its radioactivity was counted for 1 minute.

**INDIRECT MEASUREMENT OF E₁₅ BY A CONVENTIONAL METHOD**

Samples were hydrolyzed, and the released E₁ was measured as described elsewhere [4]. Briefly, 0.2 mL of serum/plasma samples supplemented with 500 cpm of tritiated E₁₅ (New England Nuclear; specific activity, 40–60 Ci/mmol) in 0.1 mL of assay buffer were incubated at 37 °C for 30 min, followed by extraction with 10 mL of ethyl acetate/hexane (4:6 by vol) to remove unconjugated steroids. The aqueous phase was then deproteinized by the addition of 2 mL of methanol. The protein pellet was removed by centrifugation, and the methanol layer was evaporated under nitrogen. The residue was reconstituted with 2.0 mL of 0.1 mol/L sodium acetate buffer (pH 5.0), and E₁₅ was hydrolyzed with aryl-sulfatase (type VII from abalone entrails, Sigma Chemical Co.) at 37 °C for 16–18 h. The hydrolyzed estrogens were

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**Fig. 1. Synthesis of estrone-3-sulfate:BSA and estrone sulfate-6-CMO-histamine.**
extracted twice with 5 mL of ethyl acetate:hexane (4:6 by vol). The solvent was evaporated, and the residue was redissolved in 1 mL of assay buffer:ethanol (9:1 by vol) and used for the indirect estimation of E1S by using a RIA for E1.

Results
Figure 1 illustrates the synthesis of E1S-6-CMO-BSA as the immunogen and E1S-6-CMO-histamine as the precursor molecule to 125I-labeled tracer, both of which were synthesized from E1S-6-CMO, which in turn was obtained via chemical sulfation of E1-6-CMO.

For the development of direct RIA for E1S, various antiserum titers, sample and tracer volumes, incubation times, and temperatures were investigated. Ideal conditions in terms of assay performance are described in Materials and Methods. With these conditions, the calibration curve was useful over the range of 0.05 to 90.0 µg/L with 50% displacement at 2.5 µg/L. A linear/log transform of a typical calibration curve is shown in Fig. 2.

Performance Characteristics
Sensitivity. The limit of detection, as determined by interpolating the mean – 2 SD of 12 replicates of the zero standard, was 0.009 µg/L.

Precision. The intraassay and interassay CVs are shown in Table 1. The intraassay variation was calculated from the 16 replicates each of the three serum samples containing various endogenous concentrations of E1S. The CVs were <10% for each sample. The interassay variation was calculated from multiple analyses (n = 8) of three different serum samples. The CVs were <10% for all samples.

Analytical recovery. Recovery experiments were carried out with serum samples containing low endogenous concentrations of E1S to which three different amounts of exogenous E1S had been added. The samples were then analyzed (Table 2). In each case, the amount recovered was >80%.

Linearity of dilution. Three serum samples with low, medium, and high endogenous concentrations of E1S were diluted serially with the zero calibrator. It showed the values between 80% and 105% of expected, and the regression analysis showed E1S (expected) = 0.93 E1S (observed) – 0.0074, r = 0.99, indicating that the assay procedure maintains good linearity of dilution.

Specificity. The cross-reactivity of the antiserum used for the development of RIA of E1S is shown in Table 3. The antiserum shows relatively low cross-reactivity to the unconjugated estrogens and nonestrogenic steroids. E1 and estrone 3-glucuronide showed <5% cross-reactivity.

Table 1. Precision of E1S RIA.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intraassay variation (n = 16)</th>
<th>Interassay variation (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD, ng/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>01</td>
<td>0.35 ± 0.03</td>
<td>9.2</td>
</tr>
<tr>
<td>02</td>
<td>8.97 ± 0.40</td>
<td>4.5</td>
</tr>
<tr>
<td>03</td>
<td>59.80 ± 2.74</td>
<td>4.6</td>
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Table 2. Mean E1S values measured in serum samples after the addition of known amounts of E1S.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous µg/L</th>
<th>Added µg/L</th>
<th>Expected µg/L</th>
<th>Observed µg/L</th>
<th>% recovery</th>
</tr>
</thead>
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<tr>
<td>01</td>
<td>0.48</td>
<td>13.05</td>
<td>13.58</td>
<td>12.12</td>
<td>89.2</td>
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<td></td>
<td>32.62</td>
<td>33.11</td>
<td>28.53</td>
<td>86.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>65.25</td>
<td>35.73</td>
<td>54.74</td>
<td>92.6</td>
<td></td>
</tr>
<tr>
<td>02</td>
<td>0.30</td>
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<td>13.35</td>
<td>13.07</td>
<td>97.9</td>
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<td></td>
<td>32.62</td>
<td>32.92</td>
<td>27.07</td>
<td>83.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>65.25</td>
<td>65.55</td>
<td>66.99</td>
<td>102.2</td>
<td></td>
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<tr>
<td>03</td>
<td>1.94</td>
<td>14.99</td>
<td>16.57</td>
<td>110.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32.62</td>
<td>34.57</td>
<td>36.39</td>
<td>105.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>65.25</td>
<td>67.19</td>
<td>70.45</td>
<td>104.8</td>
<td></td>
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</tbody>
</table>

Table 3. Percentage of cross-reactivity of various steroids with an antiserum against E1S-BSA used for RIA of E1S.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1S</td>
<td>100</td>
</tr>
<tr>
<td>E1</td>
<td>4.9</td>
</tr>
<tr>
<td>Estrone glucuronide</td>
<td>3.4</td>
</tr>
<tr>
<td>17β-Estradiol 3-sulfate</td>
<td>1.0</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>0.3</td>
</tr>
<tr>
<td>17β-Estradiol glucuronide</td>
<td>0.1</td>
</tr>
<tr>
<td>Equilin</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Equilenin</td>
<td>2.3</td>
</tr>
<tr>
<td>DHEA sulfate</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Estriol 3-sulfate</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Estriol 3-glucuronide</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Pregnanolone sulfate</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* All other related steroids showed <0.1% cross-reactivity.
COMPARISON OF DIRECT RIA WITH CONVENTIONAL METHOD FOR THE MEASUREMENT OF E₁S

E₁S values were measured in 71 serum samples by the new, direct RIA (y) and by an established, conventional method (x) that utilized hydrolysis, extraction, and subsequent E₁ measurement by RIA (thus, indirectly calculating E₁S values). Regression analysis (Fig. 3) of the samples yielded the equation:

\[ y = 1.54x - 1.43 \quad (r = 0.985, S_{yx} = 2.14, n=71) \]

Fig. 3. Comparison of serum values of E₁S obtained by new RIA and by indirect measurement of E₁S after solvolysis.

SUBSTANTIAL EQUIVALENCE BETWEEN E₁ AND E₁S

Forty-one samples were evaluated for E₁S and E₁ (DSL 8700, estrone RIA kit). The correlation coefficient was 0.86, with \( P < 0.0001 \) showing substantial equivalence of the two steroids in the same patient sample (Fig. 4).

Fig. 4. Comparison of serum values of E₁S and E₁ (DSL 8700).

COMPARISON OF SERUM VS PLASMA E₁S

Analysis of serum and plasma obtained from the same subjects (n = 32) for E₁S in the new RIA showed no significant difference in E₁S values, indicating that the assay can be used to measure E₁S concentrations in serum or plasma (r = 1.0).

E₁S IN MEN AND WOMEN

The new RIA was used to measure E₁S values in serum samples from normal, apparently healthy men, premenopausal women in their different phases of menstrual cycle or on oral contraceptive regimen, women in their first, second, or third trimester of pregnancy, and postmenopausal women with or without hormone replacement therapy (HRT; Premarin). Fig. 5 shows the E₁S concentrations in various groups of subjects.

Discussion

E₁S is quantitatively the most important circulating estrogen in a woman. Its production rate fluctuates during the menstrual cycle, similar to that observed for E₂ and E₁. Estrogen status in women is conventionally determined primarily on the basis of serum concentrations of E₂ and sometimes serum E₁. There has been considerable interest in the third serum marker, E₁S, which is biologically inactive and can be readily converted into E₁ and E₂. Because the circulating pool of E₁S can be conceptualized as a slowly metabolized estrogen reservoir, it has gained marked importance in assessing estrogenicity in a woman. However, few studies have been done on the potential diagnostic utility of E₁S \[5\], primarily because its
measurement has required cumbersome conventional methodology involving many steps of extraction, hydrolysis, and reextraction before its measurement by RIA. This poses practical limitations on the measurement of E1S. Moreover, some antibodies used to determine E1S lack specificity because they were raised against estrone glucuronide and possessed high cross-reactivity to E1S.

In the present study, the antiserum against E1S was prepared by linking the carrier protein through the 6-position in ring B of the steroid skeleton. This presumably retained the stereochemistry of critical rings A and D with the spatial conformation of sulfate group at C-3, keto group at C-17, and aromativity of ring A intact. This was reflected by the antiserum, which had high specificity for E1S, with very little or no cross-reactivity with other estrogenic as well as nonestrogenic steroids. The cross-reactivity with E1 or estrone 3-glucuronide (<5%) does not present serious problems in our assay because the concentration of E1S is severalfold higher than E1 or estrone glucuronide in the serum [1].

Performance characteristics for the RIA as determined by sensitivity, intra- and interassay precision, analytical recovery, and linearity of dilution studies were acceptable. Comparison of serum samples by using the new RIA and a conventional method for measurement of E1S showed that this new RIA does not require laborious sample pretreatment. There was excellent correlation between the two methods. However, the samples read lower with the conventional method. One possible reason could be the multiple steps involved in the conventional assay, which can result in lower recovery of E1. Excellent correlation between the paired serum and plasma samples for E1S values indicates that either one can be used in the assay. A positive correlation was observed between E1 and E1S values from the same subject, indicating the parallelism between the two estrogens in the same subject.

Earlier reports [10, 11] have indicated considerably higher serum concentrations for E1S as compared with E1 and E2. In the present study, we selected several patient categories to establish the reference range for E1S. The means, medians, and ranges for these values are presented in Fig. 5. Normal men have a low mean value of 0.96 µg/L. Follicular phase women have the same mean value, but E1S concentrations in luteal phase women are approximately doubled to 1.74 µg/L. In women on oral contraceptives, because the ovary is suppressed and the ethinyl estradiol, a component of birth control pills, is not recognized in this assay, the mean value is 0.74 µg/L. In postmenopausal women without HRT, the values are even lower, mean 0.13 µg/L, because the ovary is not producing appreciable amounts of estrogen [12]. Conversely, when postmenopausal women are on HRT, the values are markedly increased to a mean of 2.56 µg/L, indicating that estrogen from HRT continues to be sulfated even in the absence of ovarian estrogen production. The highest values for E1S are found in normal, singleton pregnant women; the values are even higher in late pregnancy, going from a mean of 19.5 µg/L in the first trimester to 66 µg/L and to 105 µg/L in the second and third trimesters, respectively. These values are essentially what would be expected from earlier reports and represent marked increases above the normal circulating concentrations of E1 and E2 [10, 11].

Monitoring E1S because of its higher concentration and slow metabolic rate in serum would make it a more reliable marker to determine estrogenicity in women. The availability of the new, direct RIA for measurement of E1S provides a valuable tool to study human and also veterinary physiology of this important marker of estrogenicity. Efforts are under way to develop a simple, nonisotopic E1S enzyme immunoassay.

References