Hypothesis/Commentary

Limitations of Direct Immunoassays for Measuring Circulating Estradiol Levels in Postmenopausal Women and Men in Epidemiologic Studies

Frank Z. Stanczyk[1,2], Jordan Jurow[1], and Ann W. Hsing[3]

Abstract

Serum estradiol (E_2) serves as an important diagnostic marker in a variety of clinical conditions. In epidemiologic studies, E_2 is commonly used to define the etiologic role of estrogen in hormone-related cancers and chronic conditions. Having an accurate and reliable E_2 assay is of critical importance in these studies, especially when measuring the very low E_2 levels (<30 pg/mL) common in postmenopausal women and men, and for discerning the relatively small (usually <20%) case-control differences in E_2 levels. Because E_2 is metabolized to >100 metabolites in the body, some of which cross-react with E_2 antibodies, direct RIAs without purification steps lack specificity for E_2 and can substantially overestimate E_2 levels. Although direct E_2 RIAs using commercial kits are simpler, less time consuming, and less expensive and require less sample volume than conventional RIAs with preceding purification steps, their lack of sensitivity and specificity makes them invalid for measuring circulating E_2 levels in epidemiologic studies of postmenopausal women or men. Instead, we recommend the use of a well-validated RIA with purification steps to improve sensitivity and specificity and to help achieve the necessary accuracy and reliability needed for epidemiologic studies.

Cancer Epidemiol Biomarkers Prev; 19(4); 903–6. ©2010 AACR.

Estradiol (E_2) in serum serves as an important diagnostic marker in a variety of clinical conditions in both men and women. Clinically, serum levels of E_2 are used to assess ovarian function in women with menstrual disorders, precocious or delayed puberty, and assisted reproduction, as well as to monitor the effect of aromatase inhibitor treatment in breast cancer patients and determine postmenopausal status. In men, serum E_2 is used to assess gynecomastia. In epidemiologic studies, circulating levels of E_2 are often used to assess the etiologic role of estrogen in hormone-related conditions, including cancers of the breast, ovary, prostate, and liver. Accurate and reliable E_2 assays are essential for the validity of such studies. This is especially important when measuring E_2 levels in postmenopausal women or elderly men whose E_2 levels are low (<30 pg/mL). The optimum assay for such measurements is gas or liquid chromatography–tandem mass spectrometry (GC- or LC-MS/MS) or RIA with preceding extraction and chromatography steps (often referred to as conventional or indirect RIA). However, direct immunoassays, which include RIA, and chemiluminescent and enzyme immunoassays without a preceding purification step, are often used in epidemiologic studies to measure E_2 levels in postmenopausal women and elderly men, although in endocrinology literature it has been established that direct immunoassays are not valid tools for the measurement of low E_2 levels (1-11). The lack of sensitivity and specificity of direct immunoassays contributes to inconsistent results in the literature.

Although use of mass spectrometry assays for measurement of steroid hormones is growing rapidly, immunoassays will continue to be used widely in studies and clinical diagnostic testing. The original gold standard RIA used to quantify serum E_2 levels was first described by Abraham in 1969 (12, 13). This particular method requires an initial purification of E_2 before quantification by RIA. The purification is achieved by organic solvent extraction and Celite or Sephadex column chromatography. The organic solvent extraction step removes conjugated steroids, such as estrone sulfate, which is present in high concentrations in women and men, whereas the chromatographic procedure separates out most of the interfering E_2 metabolites (e.g., estrone), which could cross-react

Authors' Affiliations: Departments of [1]Obstetrics and Gynecology and [2]Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California and [3]Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Bethesda, Maryland

Corresponding Author: Frank Z. Stanczyk, Keck School of Medicine, University of Southern California, Livingston Research Building, 1321 North Mission Road, Los Angeles, CA 90033. Phone: 323-226-3220; Fax: 323-226-2850. E-mail: fstanczyk@socal.rr.com

doi: 10.1158/1055-9965.EPI-10-0081

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with the antibody in the assay. Use of a highly specific E_2 antibody in the RIA step further increases the specificity of the assay. The extraction/chromatographic E_2 RIA (conventional RIA) was shown to be sensitive, precise, specific, and accurate (12, 13).

The conventional RIA was applied to other steroid hormones, and the method has remained essentially the same for ~40 years, except for use of an iodinated marker (^{125}I), instead of tritium, to provide greater assay sensitivity. Because the extraction and chromatography steps are cumbersome, time consuming, and costly, direct RIA methods without the purification steps were developed in the late 1970s to quantify steroid hormones directly. The ^{125}I-labeled marker used in RIAs was replaced with a chemiluminescent or enzymatic label to allow direct immunoassays to be automated, thereby substantially increasing the throughput of samples for steroid hormones.

Direct RIAs lack sensitivity in measuring serum levels of E_2 in postmenopausal women and men. Assay sensitivity is defined as the minimal detection limit of an assay. From the practical standpoint, it is the lowest concentration of a standard (on the standard curve) that can be distinguished from the zero standard and is based on the confidence limits of the measurements. A major deficiency is that manufacturers of kits used to measure steroid hormones seldom report how the sensitivity of their assay was determined. Most direct RIAs have a reported sensitivity ranging from 5 to 20 pg/mL, whereas E_2 levels in postmenopausal women and elderly men are usually <30 pg/mL and are often <5 pg/mL in very old women and men. Although the manufacturer of one direct E_2 RIA kit (Siemens Medical Solutions Diagnostics) indicates that their E_2 RIA has a sensitivity of 1.4 pg/mL, it should be noted that the lowest E_2 concentration used for the standard curve in the assay is 5 pg/mL. Measurements below the lowest point on a standard curve are not reliable and should not be accepted as valid data for statistical analysis.

Direct E_2 RIAs also lack specificity in measuring low E_2 levels. In the body, E_2 is converted to >100 conjugated and unconjugated metabolites. Some of the conjugated estrogens, such as estrone sulfate, have very high concentrations in the circulation and may cross-react with the antibody in the RIA, resulting in falsely high E_2 values. The lack of specificity also explains why direct assays are not valid for measuring serum E_2 levels in postmenopausal women receiving oral estrogen therapy, such as micronized E_2 or conjugated equine estrogens. Because the concentrations of the metabolites of these administered estrogens are very high, some of the metabolites will be recognized by the antibody, and if they are not removed by a purification step in the assay, this will result in gross overestimation of the E_2 levels.

Matrix differences between serum samples and the pure solutions of varying amounts of E_2 used to generate the standard curve in a direct E_2 RIA may also affect the validity of the results. This is especially true of hemolyzed and lipemic samples, in which there may be interference by RBCs and lipids, respectively, with the binding of the antigen (E_2) to the antibody.

To illustrate the point that direct RIAs are unreliable and invalid in measuring samples with low E_2 levels, we carried out a study comparing serum E_2 levels measured by direct RIA (Siemens Double Antibody Estradiol RIA kit) and our conventional RIA (10) in 38 female samples with low E_2 levels (<30 pg/mL) and 15 samples from postmenopausal women treated with

Table 1. Comparison of E_2 levels obtained by direct and conventional E_2 RIAs in serum samples in women with low E_2 values

Sample no.	Direct RIA (E_2 pg/mL)	Conventional RIA (E_2 pg/mL)
1	<5	6
2	<5	12
3	<5	9
4	<5	12
5	5	8
6	8	18
7	<5	9
8	<5	16
9	<5	14
10	6	12
11	<5	10
12	6	14
13	<5	8
14	6	10
15	<5	8
16	<5	9
17	<5	14
18	<5	9
19	<5	7
20	<5	14
21	27	23
22	27	23
23	39	20
24	<5	8
25	<5	<3
26	25	18
27	<5	15
28	7	20
29	<5	11
30	73	24
31	27	14
32	68	29
33	<5	19
34	56	21
35	<5	16
36	<5	9
37	<5	16
38	<5	9

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estrogen. As shown in Table 1, 24 (63%) of the 38 E_2 measurements by direct RIA were below the detection limit (<5 pg/mL). This shows the importance of choosing a highly sensitive assay to measure very low E_2 levels for epidemiologic studies, such as a conventional RIA or GC- or LC-MS/MS, all of which have a sensitivity of ≤2 pg/mL (11).

Of the 14 (37%) samples with detectable E_2 levels by direct RIA (Table 1), the measurement by direct RIA in six samples was lower than that by conventional RIA, whereas eight were higher, suggesting a mixed pattern of cross-reactivity and misclassification. Thus, it should be noted that when assaying samples with low E_2 levels, sensitivity and reliability are not the only concerns. Specificity should be a concern because at low levels of E_2 cross-reactivity with E_2 metabolites may result in falsely high E_2 levels that are difficult to interpret. It is also unclear whether the extent of cross-reactivity is independent of the "true" E_2 concentration and whether there is differential cross-reactivity in cases and controls.

Table 2 shows E_2 values determined by the direct and conventional RIAs in serum samples obtained from the postmenopausal women treated with estrogen (micronized E_2 or conjugated equine estrogen). As shown, a large overestimation by the direct E_2 RIA is evident. For each sample, E_2 levels measured by direct RIA are much higher than those by conventional RIA, although the magnitude of the difference is not related to baseline levels. In some samples, the measured concentrations by direct RIA are 10 times higher than those by conventional RIA, suggesting large cross-reactivity of the assay antibody with the E_2 metabolites following treatment with micronized E_2, and with metabolites of E_2 and equine estrogens (equilin, equilenin, and their metabolites) following treatment with conjugated equine estrogens. The correlation coefficient between direct and conventional RIA measurements was −0.33 (P = 0.24; Fig. 1). The manufacturer of the direct E_2 RIA kit does recommend that an organic solvent extraction step be used before measuring E_2 by the direct RIA in samples obtained from women using estrogen therapy. However, the extraction step removes only conjugated estrogens; there are still >30 unconjugated estrogens that remain in the organic layer after extraction, some of which may cross-react with the antibody in the direct RIA. Therefore, a chromatographic step should be added to the purification procedure to remove the interfering estrogen metabolites before the RIA. For similar reasons, a preceding chromatographic step is also essential when measuring E_2 levels by RIA in serum samples obtained from women with breast cancer who are undergoing treatment by aromatase inhibitors. Certain aromatase inhibitors (e.g., fulvestrant and/or their metabolites) may cross-react with the antibody in the assay when the chemical structure of the drug resembles that of E_2.[4] In such assays, there is only a partial suppression of E_2 compared with a conventional RIA, which shows high suppression of the estrogens. This may lead to erroneous clinical decisions in the treatment of breast cancer patients (14).

An important consideration in the validation of any steroid hormone RIA is assay accuracy. This is usually carried out by spiking stripped serum samples with a known amount of steroid and then measuring the steroid in the samples by RIA. One should expect the recovery of the steroid to be 90% to 110%. In our study, in stripped serum samples containing 15, 41, and 121 pg/mL of E_2 measured by our conventional RIA, the concentrations

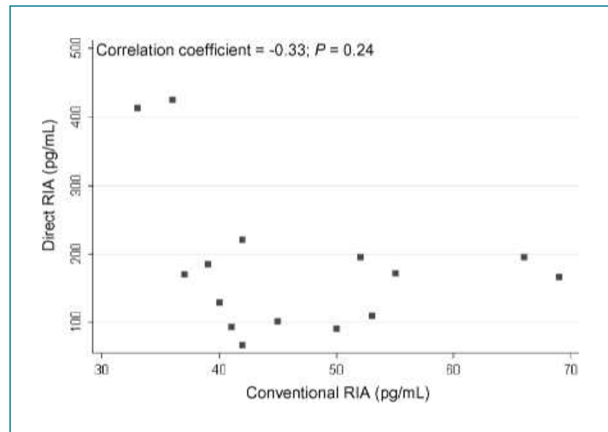

Figure 1. Correlation between E_2 levels measured by direct RIA and conventional RIA in serum from postmenopausal women (n = 15) treated with estrogen.

Table 2. Comparison of E_2 levels obtained by direct and conventional E_2 RIAs in serum samples from postmenopausal women treated with estrogen

Sample no.	Direct RIA (E_2 pg/mL)	Conventional RIA (E_2 pg/mL)
1	185	39
2	195	66
3	220	42
4	172	55
5	102	45
6	94	41
7	109	53
8	196	52
9	167	69
10	170	37
11	91	50
12	130	40
13	68	42
14	425	36
15	414	33

[4] Unpublished data.

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measured in the same samples by the Siemens direct E_2 RIA were <5, 10, and 50 pg/mL of E_2, respectively. The reason for the underestimated E_2 values measured by the direct RIA is not known but may be due to several factors, such as the presence of different populations of antibodies in the antiserum used for the RIA.

In conclusion, because of the poor sensitivity and specificity of direct immunoassays, especially at low levels of E_2, we recommend the use of conventional RIA or a GC- or LC-MS assay in epidemiologic studies measuring serum E_2 levels in postmenopausal women and men to improve the validity of the study. Use of inappropriate E_2 assays, such as direct RIA without purification, may compromise the validity of the epidemiologic research, leading to erroneous conclusions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Received 01/18/2010; revised 02/22/2010; accepted 02/23/2010; published OnlineFirst 03/23/2010.

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