Reliability of Measurements of Total, Protein-Bound, and Unbound Estradiol in Serum[1]

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Abstract

Estradiol (E_2) circulates in the blood in three states: unbound (U-E_2), bound to sex-hormone binding globulin (SHBG-E_2), and bound to albumin. There is evidence to support the concept that only U-E_2 and albumin-bound E_2, are bioavailable (i.e., rapidly extracted by tissues). A case-control study nested within a large cohort of women, in which we are examining the effect of estrogens on breast cancer risk, offered the opportunity to assess the reliability of measurements of E_2, the percentage of SHBG-E_2, and the percentage of U-E_2 based on multiple annual serum specimens. Long-term (1–2 year) reliability, as estimated by the intraclass correlation coefficient, was assessed in a subgroup of 71 premenopausal and 77 postmenopausal controls for whom two or three serum specimens were assayed. In postmenopausal women the intraclass correlation coefficient for a single measurement of total E_2 was only 0.51. As for the percentage of SHBG-E_2, intraclass correlation coefficients were 0.83 and 0.94, and for U-E_2, 0.72 and 0.77 in the premenopausal and postmenopausal groups, respectively. These data suggest that, whereas single determinations of total E_2 are insufficient to reliably estimate a woman's true mean level, a single measurement of the percentage of SHBG-E_2 or U-E_2 is adequate to assess bioavailability of E_2 in an epidemiological study, irrespective of day of the menstrual cycle.

Introduction

In normal women, approximately 98.5% of E_2[3] circulates either loosely bound to albumin (A-E_2) or tightly bound to sex-hormone binding globulin (SHBG-E_2) while the remaining 1.5% is unbound (U-E_2). The biological activity of E_2 is believed to be primarily attributable to U-E_2, which is readily available to cross cell membranes (1, 2), although there is evidence that A-E_2 is also biologically active (3). In an attempt to clarify the role of estrogens in breast cancer, several small epidemiological studies have measured the percentage of U-E_2 (and sometimes the percentage of A-E_2) rather than total E_2. Although most have reported positive, albeit weak, associations (4–11), a few have not detected appreciable differences between cases and controls (12–14).

All published studies examining the association between the percentage of U-E_2 and breast cancer have compared hormone concentrations in single specimens, without concern for the underlying fluctuations in total E_2, which are known to be substantial under normal conditions in both premenopausal and postmenopausal women (15). These fluctuations in E_2, which occur throughout the menstrual cycle and perhaps as a consequence of environmental, metabolic, or emotional stimuli, may influence the fraction of the hormone which is bound to proteins, thus introducing variability in the percentage of U-E_2. Depending on the extent of such variability, single determinations may provide an inaccurate estimate of the true average value over an extended time period, which presumably influences breast cancer risk.

During the course of a case-control study nested in a large cohort of women, in which we are examining the effect of estrogens on breast cancer risk, E_2 measurements were made on stored serum specimens which had been drawn at intervals of approximately 1 year. The availability of multiple determinations in a subset of study subjects offered us an opportunity for addressing the issue of the long term reliability of total E_2 and its percent fractions.

Materials and Methods

Study Population. Study subjects were women of all races aged 34–65 years attending a breast screening clinic in New York City who volunteered to donate blood for a cohort study of hormones, diet and cancer (16). The cohort was restricted to women not pregnant or treated with hormones in the preceding 6 months. Between March 1985 and June 1991, 14,290 women were recruited. The cohort is being followed to identify new cases of any cancer. Additional blood donations were solicited when a study member returned to the clinic for annual screening. About 51% of cohort members donated blood specimens on more than one occasion, usually at 1-year intervals. Blood was collected without eating restrictions before breast examination, between 9 a.m. and 3 p.m. Blood samples from premenopausal women were collected without regard to time of the menstrual cycle. Specimens were frozen at −80° for long-term storage soon after collection.

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[3] The abbreviations used are: E_2, estradiol; SHBG, sex-hormone binding globulin; SHBG-E_2, SHBG-bound estradiol; U-E_2, unbound estradiol; A-E_2, albumin-bound estradiol; ANOVA, analysis of variance.

Table 1 New York University Women's Health Study: Number of controls in the breast cancer nested case-control study available for assessment of E_2 reliability

No. repeat specimens	Premenopausal		Postmenopausal	
	No. of subjects	No. of matched sets	No. of subjects	No. of matched sets
2	67	17[a]	55	28[a]
3	4	1	22	12[b]
Total	71	18	77	40

[a] One incomplete matched set.
[b] Two incomplete matched sets.

Women developing an invasive carcinoma of the breast after entering the cohort are being included in a case-control study nested within the cohort. Individually matched controls are selected at random from risk sets consisting of all cohort members alive and free of breast cancer at the time of the breast cancer diagnosis of a case, and who match the case on menopausal status, age at entry, and number and approximate dates of blood donations up to the date of diagnosis of the matched case. Premenopausal subjects are additionally matched on day of menstrual cycle at the time of first blood drawing only. Two controls are selected for each postmenopausal case and four for each premenopausal case.

By September 1991, laboratory analyses of E_2 and E_2 fractions were completed on the first group of 159 cases and 428 controls. In the analyses presented here, we focused on the 71 premenopausal and the 77 postmenopausal controls with multiple measurements (Table 1). These subjects were part of 58 matched sets in the case-control study. Of the 71 premenopausal subjects, 67 had two replicates and four subjects had three, whereas of the 77 postmenopausal subjects, 55 had two replicates and 22 had three.

Laboratory Methods. All serum specimens of a case and her matched controls were analyzed in the same batch (generally on the same day) by a laboratory technician who was blind to case or control status. A batch generally included several matched case-control sets. Intra-assay variability and coefficients of variation were assessed by including blinded duplicate serum aliquots, which were always assayed within the same batch. Specimens from a standard serum pool were assayed with each batch to track the performance of the assay over time.

The assay to determine the percentage of U-E_2 was an adaptation of an ultrafiltration method previously described (17), exploiting a double isotope technique (18). The labeled materials were [2,4,6,7,16,17-^{3}H]estradiol, purified by high-pressure liquid chromatography on the day of use, and ^{14}C-glucose (DuPont NEN Research Products, Boston MA) prepared in a 9:1 cpm ratio in ethanol. With all operations at 37°, the labeled material (5 μl containing about 50,000 cpm ^{3}H) was added to 0.4 ml of test serum. After equilibration for 30 min with gentle shaking, the serum was transferred to a Centrifree Micropartition System (Amicon Corp., Beverly, MA). The ultrafiltration device was placed in a 12-slot, 6-setting Clay-Adams table top centrifuge and spun at setting 4 (1240 x g) for 1 min, yielding an ultrafiltrate of about 30 μl that was discarded. The serum was gently stirred and 30 μl was transferred to a mini-counting vial (U). Centrifugation was resumed for 3 min. After mixing, an additional 30 μl of serum was added to the original U-vial. The ultrafiltrate (approximately 50 μl) was added to a second counting vial (L). Samples U and L were assayed for ^{3}H and ^{14}C in a Beckman LS5000TD liquid scintillation spectrometer, counting for two 10 min cycles. The percentage of U-E_2 was calculated according to the formula: (^{3}H/^{14}C in L)/(^{3}H/^{14}C in U) × 100.

The assay to determine the percentage of SHBG-E_2 was performed essentially as described by Bonfrer *et al.* (19) with minor modifications. The method depends on the observation that SHBG-E_2 binds to Concanavalin A-sepharose (Sigma Chemical Co., St. Louis, MO) although A-E_2 and U-E_2 do not.

Total E_2 was measured by radioimmunoassay using an immuno-direct kit (Pantex, Santa Monica, CA). The assay is highly specific (cross-reactivity < 0.025 for estrone and estriol) and sensitive (10 pg/ml is readily detectable).

Statistical Methods. Reliability was assessed by calculating the intraclass correlation coefficient, also known as the reliability coefficient (20), using a nested ANOVA model (21). Total E_2 was transformed to the natural logarithm to normalize its positively skewed distribution. In the ANOVA model, hormone measurement was the response variable, and the independent variables were assay batch, matched set (nested within batch), and subject (nested within assay batch and matched set). The nested design permitted us to examine the effects of batch and the matching on the E_2 measurements. It also allowed us to calculate reliability coefficients which can be applied to our matched case-control results. The reliability coefficients were calculated as the between-subject variance component derived from the nested model divided by the sum of the between- and within-subject variance components. Confidence intervals for the reliability coefficients were adapted from Zar (22) for the nested design.

Results

Descriptive statistics for total E_2 and the percentage of SHBG-E_2 and U-E_2 are shown in Table 2, according to menopausal status. The total E_2 values in the premenopausal group ranged from 16.0 to 660.0 pg/ml, with a mean of 147.0. As expected, the postmenopausal levels were lower, ranging from 6.5 to 259.5 pg/ml with a mean of 38.9. The levels of the percentage of SHBG-E_2 were very similar in the two groups. The mean percentage of SHBG-E_2 was 43.8 (range, 13.7–64.9) in premenopausal subjects and 45.7 (range, 16.0–69.5) in the postmenopausal. Similarly, mean percentages of U-E_2 were 1.31 (range, 0.75–2.03) and 1.34 (range, 0.60–2.58) in the premenopausal and postmenopausal groups, respectively. Results for the percentage of SHBG-E_2 and U-E_2 corresponded to the values that have been reported in the literature (17, 19).

Estimates of variance components and reliability coefficients are also reported in Table 2. Since multiple blood specimens in the premenopausal subjects had been taken at different parts of the menstrual cycle, it was not possible to compute reliability estimates which were controlled for day of cycle. Therefore, we were unable to compute a meaningful estimate of the reliability of total E_2 measurements in premenopausal women, since E_2 levels vary greatly across the menstrual cycle. (The uncontrolled reliability estimates for total E_2 in our premenopausal group was zero). In the postmenopausal group, the within-subject variance of total E_2 was nearly as large as the between-subject variance, and this was reflected in the estimated reliability coefficient,

Table 2 New York University Women's Health Study, breast cancer nested case-control study: levels of total E_2 and E_2 protein-bound fractions, variance components, intraclass correlations and coefficients of variation among 71 premenopausal and 77 postmenopausal controls with repeat blood sampling

	Total E_2[a] (pg/ml)		SHBG-E_2 (%)		U-E_2 (%)	
	Pre	Post	Pre[b]	Post	Pre[b]	Post
Mean[c]	147.0	38.9	43.8	45.7	1.31	1.34
Median[c]	115.8	31.5	44.7	46.8	1.29	1.33
Variance components:						
Between subjects	ND[d]	0.189	78.8	130.7	0.033	0.044
Within subjects	ND[d]	0.179	15.6	8.4	0.013	0.013
Intraclass correlation	ND[d]	0.51	0.83	0.94	0.72	0.77
95% CI[e]		(0.27, 0.72)	(0.73, 0.90)	(0.90, 0.97)	(0.57, 0.83)	(0.63, 0.88)
Coefficient of variation (%)[f]	2.4	9.3	2.9	4.6	6.1	5.0

[a] Variance components, coefficients of variation and intraclass correlations for total E_2 were computed on the natural log scale.
[b] Premenopausal estimates are not controlled for day of the menstrual cycle.
[c] Mean and median of individual subject means.
[d] Not determined.
[e] Confidence interval.
[f] Within assay.

which was only 0.51. On the contrary, the within-subject variances of the percentage of SHBG-E_2 and U-E_2 were small relative to the between-subject variances in both menopausal groups. Intraclass correlation coefficients were high, 0.83 and 0.94, for the percentage of SHBG-E_2, and somewhat lower, 0.72 and 0.77, for the percentage of U-E_2. Reliability did not change in variance component analyses restricted to subgroups of time since menopause (<2 years, >2 years).

Degradation of specimens during long-term freezer storage seems an unlikely explanation for lack of reliability of total E_2. Plots of total E_2 and percentage SHBG-E_2 and U-E_2 *versus* length of time in storage did not reveal evident downward or upward trends, and slope estimates were all close to zero (data not shown).

The intra-assay coefficients of variation were below 10% for all assays (Table 2), thus indicating good within-batch reproducibility of the laboratory determinations on repeated measurement of the same material.

Plots of measured values from the standard pools over time revealed systematic trends in the measurements for all the assays under study, and the nested ANOVA results confirmed the presence of significant batch effects. By employing a matched design in our case-control study and assaying all samples from a matched set in the same batch, we will be able to eliminate the effect of these temporal trends from our case-control comparisons. This is an important advantage of using a matched design.

Discussion

Our data confirm previous reports that a single blood measurement of total E_2 provides an unreliable estimate of an individual's true average level of this hormone over a 1- or 2-year time period. In postmenopausal women, three samples are needed to raise the intraclass correlation coefficient of the geometric mean total E_2 to 0.75 (20), a value within the range of reliabilities we found for the percent E_2 fractions.

We have shown that in spite of the large underlying variability in total E_2, percent levels of SHBG-E_2 are remarkably stable in the same individual over a 1- to 2-year period as compared with the variability observed between subjects. Percent levels of U-E_2 were also quite stable. These results are even more remarkable if one considers that premenopausal blood sampling was performed irrespective of the phase of the menstrual cycle. Moreover, the premenopausal group included a few perimenopausal women who had crossed into menopause during the study.

Our observations on the stability of the percentage U-E_2 are consistent with previous findings by other investigators (23, 24) who had shown that the percentage of U-E_2 remains constant throughout the menstrual cycle and had suggested that the percentage of U-E_2 is influenced more by SHBG concentrations than circulating steroid hormone levels. Starting with the classical work of Anderson (1), it has been assumed generally that SHBG, a glycoprotein secreted by the liver with a high affinity for specific sex steroids, is regulated by an estrogen/androgen balance by which plasma concentrations are increased by E_2 and are reduced by testosterone, most likely via a direct effect on hepatic synthesis. However, the primacy of sex steroids in SHBG regulation is questionable. Growth hormone, prolactin, insulin, somatomedin-C, and thyroid hormones, as well as nutritional and metabolic factors, have been suggested as the primary homeostatic mechanisms involved in the control of circulating SHBG concentration, along with the E_2/testosterone balance (25–27). Thus, if circulating levels of E_2 are only a minor factor in the regulation of SHBG levels, the percentage of hormone that is bound to SHBG should be influenced minimally by the hormone's short-term fluctuations. If the binding of E_2 to SHBG is the product of complex physiological and metabolic processes that are characteristic of a particular individual in a particular phase of her life, the percentage of E_2 that is bound to SHBG may be a stable characteristic of that individual for as long as her homeostatic balance is maintained, as our data seem to indicate.

We conclude that whereas a single determination of total E_2 is insufficient to reliably estimate a woman's true average level during a period of 1–2 years, single measurements of the percentage of SHBG-E_2 or U-E_2 are likely to be sufficient to define her E_2 bioavailability status. For premenopausal women, measurements of the percentage of SHBG-E_2 or U-E_2 can be made without regard to time in the menstrual cycle. These results have implications for the design of prospective cohort studies aiming at evaluating the role of E_2 fractions in hormone-dependent cancers.

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