Quantifying Estrogen Metabolism: An Evaluation of the Reproducibility and Validity of Enzyme Immunoassays for 2-Hydroxyestrone and 16α-Hydroxyestrone in Urine

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Rapid and simple enzyme immunoassays (EIAs) were recently developed to measure 2-hydroxyestrone and 16α-hydroxyestrone in unextracted urine. The balance between these competing estrogen metabolism pathways may serve as a biomarker of breast cancer risk. Before testing these assays in epidemiologic studies, we evaluated their reproducibility, and validity relative to gas chromatography–mass spectroscopy (GC–MS). Overnight 12-hr urine collections from five midfollicular premenopausal women, five midluteal premenopausal women, and five postmenopausal women were aliquoted and stored at −70°C. Two aliquots from each woman were assayed with the EIAs in a random, blinded order, monthly over 4 months and 1 year later. Reproducibility over 4 months was good for both metabolites in premenopausal women (coefficient of variation = 8–14%) and satisfactory in postmenopausal women (19%). Reproducibility over 12 months remained good in premenopausal women, but was poor in postmenopausal women, with mean readings increasing 50 to 100%. Wide variation in estrogen metabolite levels enabled a single EIA measurement to characterize individual differences among premenopausal women in midfollicular (intra-class correlation coefficient = 98–99%) and midluteal phase (85–91%). A narrower range in metabolite levels among postmenopausal women reduced discrimination (78–82%). The correlation between EIA and GC–MS measurement was excellent for both metabolites (r > 0.9), except for 2-hydroxyestrone in postmenopausal women (r = 0.6). Analysis of absolute agreement suggested that both EIAs were less sensitive than GC–MS, and each detected nonspecific background. The low concentration of estrogen metabolites in urine from postmenopausal women may explain the problems with reproducibility and validity in this menstrual group. Accordingly, more sensitive EIAs have been developed and are now being evaluated. — Environ Health Perspect 105 (Suppl 3):607-614 (1997)

Key words: breast cancer, estrogen, estrogen metabolites, reproducibility, validity

Introduction

Experimental, epidemiologic, and clinical evidence strongly suggests that endogenous estrogens influence breast carcinogenesis although the active form(s) of estrogen and the specific mechanisms remain unclear (1). In 1982, the relative importance of two major, mutually exclusive pathways for estradiol oxidation, 2-hydroxylation and 16α-hydroxylation, was postulated to determine a woman’s risk of breast cancer, based on the finding that estrogen 16α-hydroxylase activity was increased in women with breast cancer (2). Over time, the ratio of 2-hydroxyestrone to 16α-hydroxyestrone (the 2/16 ratio) has been emphasized as an indicator of the balance between the two pathways and of reduced breast cancer risk. Estradiol is first reversibly converted to estrone by 17β oxidation; most of the estrone is then irreversibly oxidized to 2-hydroxyestrone or 16α-hydroxyestrone, the initial metabolites formed along these two pathways. Recently, alteration of the balance between these two metabolic pathways has been proposed as the mechanism by which certain pesticides, herbicides, plastics, and other xenoestrogens (foreign estrogens) may increase the risk of breast cancer (3,4) and by which a low-fat diet (5) or indoles (6,7) may decrease the risk.

In vivo studies, using cell cultures and organ explants, and animal studies indicate that 16α-hydroxyestrone is a potent estrogen, genotoxic, and tumorigenic and that 2-hydroxyestrone is a weak estrogen and an estrogen antagonist (8,9). Evidence in humans is more limited. Breast tissue from breast cancer patients had nearly 5 times more 16α-hydroxylase activity than comparable tissue from women without cancer (10). A metabolic study in humans demonstrated that a decrease in dietary fat decreased 16α-hydroxylated estrogens in the urine (5), although studies of urinary estrogen profiles in vegetarians and omnivorous women and in women with breast cancer indicated the relationships are complex (11–13). To our knowledge, the relationships between breast cancer risk and 2-hydroxyestrone and 16α-hydroxyestrone, and the two metabolic pathways they represent, have not yet been examined in a case-control or cohort study, primarily because of the difficulty of assessing the two pathways in humans using radiolabeled tracer or gas chromatography–mass spectroscopy (GC–MS) methods.
Recently, rapid and simple enzyme immunoassays (EIAs) were developed to evaluate the balance between these two metabolic pathways, and possibly to serve as a biomarker of breast cancer risk (14). The EIAs measure 2-hydroxyestrone and 16α-hydroxyestrone in unextracted urine. The initial studies that examined sensitivity, specificity, coefficients of variation, recovery, and validation, relative to a GC–MS method, have been published (14).

We hope to use these EIAs in a case-control study of breast cancer in Asian-American women as one of many measures of endogenous hormone levels and hormone metabolism that might explain why breast cancer incidence rates have historically been 4 to 7 times higher in the United States than in Asia (15). This large, population-based case-control study of breast cancer, conducted among women of Chinese, Japanese, and Filipino ethnicity living in San Francisco–Oakland and Los Angeles, California, and Oahu, Hawaii, was designed to take advantage of the diversity in risk and lifestyle in these ethnic populations and to elucidate the role of modifiable exposures, related to lifestyle or environment, in the etiology of this disease (15). In preparation for this effort, we examined the reproducibility and validity of a number of hormone assays that we wish to use with the plasma and urine samples collected in the study.

Methods

Collection and Distribution of Samples

Overnight 12-hr urine samples for the hormone assays were collected from 15 volunteers working at the National Cancer Institute, Bethesda, Maryland. Midluteal phase urine was obtained 6 to 10 days after the start of menses from five premenopausal women with regular menstrual cycles (mean age = 40 years). Midluteal phase urine was collected 4 to 6 days prior to the estimated start of the next menses from five premenopausal women with regular cycles (mean age = 39 years); subsequent follow-up confirmed the timing of the urine collection. Urine was also collected from five postmenopausal women who had experienced natural menopause (mean age = 56 years); at least three years had elapsed since their last menstrual cycle. None of the 15 women was currently taking exogenous estrogens.

The 12-hr urines were collected in half-gallon plastic jugs, containing one teaspoon of boric acid to acidify the urine and to prevent bacterial growth. The urine was kept at −4°C with ice packs or refrigeration until aliquotted the next day. Each urine sample was decanted from any residue, carefully mixed, and aliquotted into 10 ml portions in 15 ml conical tubes for storage at −70°C.

During March to July 1994, Bradlow’s laboratory received four batches of urine aliquots, with one batch to be assayed at the beginning of each of 4 consecutive months. Each batch contained two aliquots from each of the 15 subjects. Within a batch, the 30 aliquots were randomly ordered, with a different order for each of the four batches. A year later, in March 1995, Bradlow’s laboratory received a fifth batch of randomly ordered aliquots, this time with only one aliquot from each of the 15 subjects. Aliquots were shipped to the laboratory in identical, sequentially numbered vials. Laboratory personnel were told only whether an aliquot was from a premenopausal or postmenopausal woman.

Also in the spring of 1995, Adlercreutz’s laboratory received a single batch of urine aliquots, containing one aliquot from each of the 15 subjects in a random order. Laboratory personnel were informed whether an aliquot was from a midluteal premenopausal, a midluteal postmenopausal, or a postmenopausal woman.

Laboratory Methods

In Bradlow’s laboratory, 2-hydroxyestrone and 16α-hydroxyestrone were measured directly and concurrently in urine with a newly developed, commercially available EIA kit (Estramet 2/16, Immuna Care Corporation, Bethlehem, PA) (14). High affinity, specific murine monoclonal antibodies for each metabolite are bound to microtiter plates, and the enzyme alkaline phosphatase is linked to each metabolite. The metabolite in the sample to be assayed competes with the metabolitealkaline phosphatase to bind to the immobilized antibody. The rate of p-nitrophenol hydrolysis is inversely related to the concentration of the metabolite in the sample.

Initially in Bradlow’s laboratory, the urine aliquots were thawed and incubated with β-glucuronidase/sulfatase to hydrolyze estrogen glucuronides and sulfates. Each urine aliquot was assayed in triplicate and the results averaged. Standards of 0.625 to 40.0 ng/ml (2.2–140 nmol/liter) were routinely used (14). For aliquots whose values fell off the standard curve, either twice the volume or a dilution of the urine was assayed. The between- and within-assay coefficients of variation for 2-hydroxyestrone, 16α-hydroxyestrone, and their ratio with this EIA kit have been reported to be consistently less than 9% (14).

In Adlercreutz’s laboratory, GC–MS was used to characterize the estrogen profile in urine after hydrolysis of conjugates (16). Fourteen endogenous estrogens—estrone, estradiol, estriol, 2-hydroxyestrone, 2-hydroxyestradiol, 2-methoxyestrone, 2-methoxyestradiol, 4-hydroxyestrone, 15α-hydroxyestrone, 16α-hydroxyestrone, 16β-hydroxyestrone, 16-β-ketoestradiol, 16-epiestriol, and 17-epiestriol—were measured, along with four lignans and four isoflavonoids. Each urine aliquot was assayed in duplicate and the results averaged. Briefly, after protection of the carbonyl functions with O-ethylhydroxylamine, estrogen conjugates were extracted on Sep-Pak C18 cartridges (Waters Assoc., Milford, MA) and purified on the acetate form of DEAE–Sephadex. The aliquots were subsequently hydrolyzed with Helix pomatia juice and the hydrolysate purified on the acetate form of QAE–Sephadex. Recovery after hydrolysis has been estimated to be 75 to 82%, based on addition of deuterated (d4)-ethoxy derivatives of all ketonic estrogens as internal standards immediately before this step (17). Estrogens with vicinal cis-hydroxyls and di phenolic compounds were fractionated on the borate and bicarbonate forms of QAE–Sephadex, respectively. Neutral steroids were removed by the free base form of DEAE–Sephadex, after which estrogens were separated into two groups using Lipidex 5000 in a straight phase system. Following trimethylsilyl ether derivatization, estrogens were analyzed by capillary gas chromatography with stable isotope dilution mass spectrometry. Deuterated internal standards were available for all the estrogens except 16α-hydroxyestrone and 17-epiestriol, and were used to correct for losses after the initial hydrolysis step. The limit of detection was estimated to be 0.5 to 3 nmol/liter. The coefficients of variation in premenopausal urine samples for the 10 major estrogens, including 2-hydroxyestrone and 16α-hydroxyestrone, have been reported to be 4 to 7% (16,17).

Statistical Methods

The means of the triplicate EIA readings for each aliquot were analyzed on the log scale (base 10) to reduce the dependence of the standard deviation of the response on the mean response. The transformation is also appropriate because studies of cancer
association typically regress log (relative risk) on the log of assay results.

For each group of women classified by menstrual phase, we estimated components of variance among women ($\sigma^2_1$), among months of analysis for a given woman ($\sigma^2_2$), and among aliquots for a given month ($\sigma^2_3$). Estimation was based on restricted maximum likelihood using the SAS procedure VARCOMP. With $y_{ijk}$ denoting the log$_{10}$ of the mean assay measurement over triplicates for woman $i$, month $j$, and aliquot $k$, the model is

$$y_{ijk} = \mu + a_i + b_{j(i)} + e_{k(i)}$$

where $a_i$, $b_{j(i)}$, and $e_{k(i)}$ are independent normal variates, each with mean zero and respective variances $\sigma^2_1$, $\sigma^2_2$, and $\sigma^2_3$. The value of $b$, the month term, is taken to be different for each woman and the same for all assays in that month for that woman. The term for month is said to be nested within woman. This nesting is denoted with $j(i)$. Similarly the effect of aliquot is nested within both month and woman, and this is denoted $k(i)$. The underlying mean is $\mu$ and is regarded as a constant.

Under the model, two assay results from woman $i$ done in different months will include the same parameter $a_i$; but the terms for month and aliquot will be different and independent. Thus, the covariance between measurements arises only from the term due to the individual. The intraclass correlation coefficient is $\sigma^2_1/(\sigma^2_1 + \sigma^2_2 + \sigma^2_3)$, i.e., the individual variance component divided by the sum of all variance components. Coefficients of variation measure the variability associated with a laboratory and are usually estimated by repeatedly assaying aliquots from a single large pool and dividing the standard deviation of the measurements by the mean value. Here the coefficient of variation was obtained by an application of the delta method, as in Gail et al. (18). We approximated the coefficient of variation by

$$100 \times 2.303 \times (\sigma^2 + \sigma^2)^{1/2}.$$
metabolites, between-individual variability was highest for the midfollicular premenopausal women and lowest for the postmenopausal women. For women in each menstrual group, between-individual variation appeared greater for 2-hydroxyestrone than 16α-hydroxyestrone.

Apart from the unusually low readings for both metabolites for one aliquot at month 3 from a midluteal premenopausal woman, a single EIA measurement, in general, reliably characterized each premenopausal woman. To see this, note that the lines connecting the midpoints of the two readings in each month for each woman are usually clearly separated (Figures 1 and 2). However, for the postmenopausal women, single EIA readings were not as reliable for characterizing an individual woman; in particular, the midpoint lines cross one another. Among the postmenopausal women, a narrower range in metabolite values and, to a lesser extent, the increased assay variability contributed to the overlapping lines and the less definitive discrimination between women.

For both 2-hydroxyestrone and 16α-hydroxyestrone, the mean of the eight EIA measurements performed on urine from each woman in the spring of 1994 was compared with the single repeat measurement made a year later (Figure 3). Readings for both estrogen metabolites increased, with the most striking effect seen in the postmenopausal women. Mean 2-hydroxyestrone readings rose in two of the midfollicular premenopausal women, in five of the midluteal premenopausal women, and in five of the postmenopausal women, while 16α-hydroxyestrone readings rose in two of the midfollicular premenopausal women, in four of the midluteal premenopausal women, and in all five of the postmenopausal women. With few exceptions, rankings among women were maintained. The mean relative increase for each estrogen metabolite was moderate but not statistically significant for the midfollicular premenopausal women, modest for the midluteal premenopausal women, and substantial and statistically significant for the postmenopausal women (Table 1).

The reproducibility of EIA measurement of 2-hydroxyestrone and 16α-hydroxyestrone is summarized and quantified in Table 1. The coefficient of variation over a 4-month interval, a measure of laboratory variability, was comparable for the two estrogen metabolites and highest in the postmenopausal women (19%), the menstrual group with the lowest concentration of both metabolites. Laboratory variation from month to month during the 4-month interval was no greater than expected, based on laboratory variation in a single day (results not shown). The intraclass correlation coefficient, the proportion of the variation in assay results attributable to differences between women, was lower in midluteal premenopausal women than in midfollicular premenopausal women, and was lowest in postmenopausal women (Table 1). In addition, it was slightly greater for 2-hydroxyestrone than 16α-hydroxyestrone. The intraclass correlation coefficient approached 99% for each metabolite in midfollicular premenopausal women (approximately 10-fold range in metabolite concentrations among the women) and dropped to approximately 80% in postmenopausal women (approximately 3-fold range in metabolite concentrations).

### Biochemical Validity of EIAs for 2- and 16α-Hydroxyestrone

An assay may be highly reproducible but not valid. For example, the assay may detect compounds structurally similar to the analyte of interest (lack of specificity). Because of its ability to separate and identify very similar compounds (excellent specificity), GC–MS is widely accepted as a gold standard for measurement of steroid hormones. The correlations between EIA and GC–MS measurement of 2-hydroxyestrone, 16α-hydroxyestrone, and the ratio of 2-hydroxyestrone to 16α-hydroxyestrone

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**Table 1. Reproducibility of EIA measurement of urinary 2-hydroxyestrone and 16α-hydroxyestrone.**

| Estrogen metabolite                        | Mean of five women | Coefficient of variation over 4 months | Intraclass correlation coefficient over 4 months | Relative increase over 1 year, % |
|--------------------------------------------|--------------------|---------------------------------------|------------------------------------------------|---------------------------------|
| Midfollicular premenopausal women          |                    |                                       |                                                |                                 |
| 2-Hydroxyestrone                           | 44.2 (10.6–116.0) | 7.8                                   | 0.99                                           | 20                              |
| 16α-Hydroxyestrone                         | 13.6 (3.2–28.7)    | 11.1                                  | 0.98                                           | 25                              |
| Midluteal premenopausal women              |                    |                                       |                                                |                                 |
| 2-Hydroxyestrone                           | 19.4 (7.5–35.8)    | 13.7a                                 | 0.91                                           | 6                               |
| 16α-Hydroxyestrone                         | 10.3 (6.0–16.9)    | 8.9a                                  | 0.85                                           | 5                               |
| Postmenopausal women                       |                    |                                       |                                                |                                 |
| 2-Hydroxyestrone                           | 5.6 (3.3–9.1)      | 18.7                                  | 0.82                                           | 57*                             |
| 16α-Hydroxyestrone                         | 2.4 (1.8–4.1)      | 18.5                                  | 0.78                                           | 105*                            |

* p ≤ 0.01. **Numbers in parentheses are ranges of individual means. ***Excludes the unusually low reading at month 3 for one aliquot from a midluteal premenopausal woman. Including these low values, the coefficients of variation for 2-hydroxyestrone and 16α-hydroxyestrone would be 19.2 and 17.3%, respectively.

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**Figure 3. Reproducibility over 1 year of EIA measurement of urinary 2-hydroxyestrone and 16α-hydroxyestrone.** The 1994 values are the grand means of the eight aliquots measured for each woman during March to July 1994 in the 4-month reproducibility study. The 1995 values are for a single aliquot from each woman measured in March 1995. Different symbols (*, ▲, ○, ▼, □) distinguish the five midfollicular premenopausal women, the five midluteal premenopausal women, and the five postmenopausal women.
Table 2. Correlation of EIA and GC–MS measurement of 2-hydroxyestrone, 16α-hydroxyestrone, and the ratio of 2 hydroxyestrone to 16α-hydroxyestrone.

| Estrogen metabolite            | Spearman rank order correlation | Pearson correlation |
|--------------------------------|---------------------------------|---------------------|
| Premenopausal women            |                                 |                     |
| 2-Hydroxyestrone               | 0.952**                         | 0.943**             |
| 16α-Hydroxyestrone             | 0.952**                         | 0.961**             |
| 2/16 Ratio                     | 0.842**                         | 0.952**             |
| Postmenopausal women           |                                 |                     |
| 2-Hydroxyestrone               | 0.600                           | 0.601*              |
| 16α-Hydroxyestrone             | 0.900**                         | 0.914**             |
| 2/16 Ratio                     | 0.600                           | 0.848**             |

*p < 0.05, **p < 0.01. The EIA measurement of a single aliquot from each woman in the spring of 1995 was compared with the GC–MS measurement of a single aliquot also obtained in the spring of 1995. Since the EIA for 2-hydroxyestrone measures other C-2 hydroxylated estrogens (13), GC–MS estimates for 2-hydroxyestrone and 2-hydroxyestradiol were combined. □ includes the midfolicular and midluteal premenopausal women.

Biologic Significance of the 2/16 Ratio

Bradlow and colleagues have suggested that the 2/16 ratio, the ratio of 2-hydroxyestrone to 16α-hydroxyestrone, reliably characterizes the relative importance of the two dominant pathways for estrogen metabolism—2-hydroxylation and 16α-hydroxylation (Figure 5). To evaluate this hypothesis, we assumed that 2-hydroxyestrone and 2-methoxyestrone are the major metabolites on the 2-hydroxylation pathway and that 16α-hydroxyestrone, 17-epiestriol, and estriol are the major metabolites on the 16α-hydroxylation pathway (PK Sihveri, personal communication.) There are differences of opinion among endocrinologists about which estrogen metabolites are the dominant ones, and whether the same pattern is seen in all women. Using the GC–MS results for all 15 women, the ratio of 2-hydroxyestrone to 16α-hydroxyestrone (the 2/16 ratio) was then compared with the ratio of (2-hydroxyestrone + 2-methoxyestrone) to (16α-hydroxyestrone + 17-epiestriol + estriol). The correlation was excellent (Spearman correlation coefficient = 0.90; Pearson correlation coefficient = 0.96) and is shown in the scatter plot in Figure 6A. In addition, the 2/16 ratio seemed consistently predictive; it reflected the relative importance of the competing pathways for the women in each menstrual group.

In the late 1970s, Lemon and colleagues (19,20) hypothesized, on the basis of mammary carcinogenesis studies in rodents, that women with a low ratio of estradiol to estrone and estradiol combined would have a high risk of breast cancer. The hypothesis was thought to explain the protective effect of full-term pregnancies (estriol) is extremely high relative to the other two estrogens in the second half of

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pregnancy) and the higher incidence of breast cancer in Western than in Asian countries (27). However, in several case-control studies, urinary levels of these estrogens were determined and the calculated ratio was not convincingly related to breast cancer risk (21,22). Pike has suggested that the 2/16 ratio should be inversely related to the ratio estriol/(estrone + estradiol) and, therefore, the early epidemiologic studies refuting the estriol/(estrone + estradiol) hypothesis from the 1970s would also tend to refute the more recent 2/16 hypothesis (MC Pike, personal communication). In Figure 6B, these two ratios are compared. The correlation was only moderate (Spearman correlation coefficient = -0.65; Pearson correlation coefficient = -0.64) and was not clearly seen in all three menstrual groups.

Recently, Adlercreutz et al. (23) emphasized the evidence, derived from in vitro studies and possible mechanisms, that estrogens hydroxylated at the 2 and 4 positions may be carcinogenic. When they compared the urinary excretion of estrogen metabolites in 12 premenopausal Finnish women and 13 premenopausal recent Asian migrants to Hawaii (who would be expected to have a substantially lower risk of breast cancer than the Finnish women), excretion of 16α-hydroxylated estrogens was similar in both groups. Moreover, the significantly greater urinary excretion of total estrogens in the Finnish women was reflected in substantially elevated urinary levels of 2- and 4-hydroxylated estrogens. Total estrogen exposure has long been postulated as an underlying hormonal cause of breast cancer (1). Thus, we examined the relationship between the 2/16 ratio and total urinary estrogens (Figure 6C). The correlation among the 15 women in our study was poor (Spearman correlation coefficient = 0.48; Pearson correlation coefficient = 0.56).

**Discussion**

To summarize, laboratory reproducibility of the EIAs for 2-hydroxyestrone and 16α-hydroxyestrone was good for both metabolites in premenopausal women (coefficient of variation = 8–14%) and satisfactory in postmenopausal women (coefficient of variation approximately 19%) over a 4-month interval. Laboratory reproducibility over a 12-month interval continued to be good for both metabolites in premenopausal women, but was noticeably poor in postmenopausal women, with the mean readings increasing 50 to 100%. Laboratory reproducibility was similar for the two metabolites. The sudden development of a problem at 12 months, which was not apparent during monitoring over 4 months, suggests that assay calibration, rather than analytic stability, was involved.

Wide variation in estrogen metabolite levels among the women enabled a single EIA measurement to characterize individual
was greater for 2-hydroxyestrone than for 16α-hydroxyestrone.

The correlation of EIA measurement with determination by GC–MS—the gold standard—was excellent \( (r > 0.9) \) for both estrogen metabolites, except for 2-hydroxyestrone in postmenopausal women. Correlations for the 2/16 ratio were no better than those for the individual metabolites, and thus also lowest for the postmenopausal women. Absolute agreement between the two measurement techniques was evaluated only in the premenopausal women. The EIAAs for both metabolites were less sensitive than GC–MS, and detected nonspecific background. The nonspecific background noise tended to cancel out for the 2/16 ratio.

Thus, this EIA kit seemed to be the least useful for postmenopausal women. First, assay reproducibility, particularly long-term reproducibility, was poor in postmenopausal women, possibly due to the low concentration of both metabolites. A moderate change over time in the nonspecific background picked up by the EIAAs would be the most problematic at low concentrations of metabolite. In addition, limited between-individual variation in absolute levels of both estrogen metabolites, compounded by poor reproducibility, caused the EIAAs to be least able to discriminate between individuals among the postmenopausal women. Finally, the correlation between EIA and GC–MS measurements diverged the most in postmenopausal women, possibly due to the low levels of metabolite relative to background noise. In response to these concerns, new, more sensitive EIAAs have been developed by TL Klug of Immunacare Corporation (Bethlehem, PA), and described by Bradlow (24). We are now evaluating the reproducibility and validity, relative to GC–MS, of these EIAAs.

Certain of our conclusions were based on small numbers; in particular, our assessment of validity was based on a total of 15 women, with 5 in each menstrual group. A larger sample of women would also have strengthened our estimate of between-individual variation in levels of these two estrogen metabolites and its magnitude relative to laboratory variation.

In addition, we had no information on within-individual variation in levels of these metabolites—the degree to which different days of the menstrual cycle, menopausal status, and age may affect estrogen metabolite levels. Within-individual variability, as well as laboratory variability, must be small relative to between-individual variability for an assay to reliably characterize an individual.

Our evaluation of the EIAAs for 2-hydroxyestrone and 16α-hydroxyestrone was unusually demanding. Most of the recent published articles on hormone measurement techniques have evaluated only reproducibility, not validity (25–28). In addition, measurement by GC–MS may not be absolutely correct. We did not test the reproducibility of the GC–MS technique. Adlercreutz et al. (11) estimated that probably 5%, and possibly as much as 10%, of the hormone metabolites might have been lost in the GC–MS analysis because of incomplete hydrolysis prior to the introduction of deuterated internal standards.

In evaluating the validity of these EIAAs, our focus was on biochemical validity, based on the published specifics of the two monoclonal antibodies. However, our ultimate interest is in biologic validity—the ability of this EIA kit, and any subsequent modifications of this kit, to predict risk of breast cancer in individual women. Even if the two antibodies are not totally specific for 2-hydroxyestrone and 16α-hydroxyestrone and cross-react with other 2-substituted and 16α-substituted estrogen metabolites, respectively, the EIAAs may still provide meaningful measures of the competition between these two metabolic pathways. Thus, we plan to evaluate these EIAAs in a case–control study of breast cancer. By focusing attention on patterns of hormone metabolism, not just absolute hormone levels, this kit suggests new approaches for studies of endogenous hormones and breast cancer.

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