The Use of Biochemical Assays in Epidemiologic Studies of Reproduction

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Recent progress in the assay of urinary hormones has opened new opportunities for epidemiologists to study hormones and health outcomes. This is especially true for studies of female reproduction. The cyclic nature of female reproduction can be fully described only by continuous frequent measurements that, in order to be practical, require easily collected biological specimens. We describe our experience in collecting and analyzing daily urine specimens from 301 healthy women. We conclude that this approach is not only feasible but potentially of great value to epidemiologists for studying fertility, early pregnancy, the effects of toxic exposures on reproduction, and the relationships between reproduction and later risk of chronic diseases.

Introduction

A woman’s reproductive hormones affect not only her ability to bear children, but also her eventual risk of osteoporosis, cardiovascular disease, breast cancer, and other chronic diseases. This interplay of reproductive hormones, pregnancy, and later illnesses is not easily studied. For example, the risk of breast cancer increases for women who have their first pregnancy after age 25 (1). There may be a hormonal mechanism responsible for this association, but the pathway of effect remains elusive despite recent progress (2,3).

Epidemiologists use data on the number and timing of births as an indirect measure of female reproductive function. Data on the occurrence of births are easily available. Furthermore, pregnancy is an important hormonal event in its own right. However, as a surrogate for a woman’s underlying hormone physiology, the counting of births has limitations. Births are an expression not only of biological capacity but of sexual and contraceptive practice. Also, births represent an unknown fraction of all conceptions. Menstruation, ovulation, and conception are by comparison much more direct measures of a woman’s reproductive physiology, but these can be difficult (or impossible) to observe. Actual reproductive hormone data from healthy women are scarce.

Advances in the technology of hormone assays are opening new opportunities for epidemiologists to observe endocrine events. In this paper we describe our experience in collecting daily urine specimens from healthy women and assaying those urines for human chorionic gonadotropin (hCG), human luteinizing hormone (hLH), and the metabolic products of estradiol and progesterone. We show the feasibility of using hormone assays to reconstruct the follicular, ovulatory, and luteal phases of the menstrual cycle and the events of early pregnancy. These methods may prove useful not only for understanding human conception and early development, but also for studying the effects of toxins on reproduction and for clarifying relations between a woman’s reproductive function and her later risk of chronic diseases.

Collection of Daily Urine Specimens

We began in 1982 to recruit healthy women into studies that required collection of daily first-morning urine specimens for up to 6 months. To our knowledge, no one had previously attempted daily urine collection from healthy volunteers over an extended period of time.

In order to maximize cooperation, we arranged to pick up urine specimens at the volunteer’s home on a schedule convenient to her, usually every 1 or 2 weeks.

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Women were fully informed of the purpose of the studies and the importance of daily urine collection. Volunteers were paid $10 a week for the collection and storage of these specimens.

Women stored specimens in their home freezers. Small coolers with blue ice (refrigerant packs) were provided to women for cold storage of specimens during vacations or other times when volunteers did not have access to a freezer. After pick-up, urines were kept frozen at $-20^\circ\text{C}$, except during thawing for assays. Time intervals between collection and analysis varied from a few months up to 4 years.

Women were asked to make daily notes on a record card at the time of each urine collection. Women indicated whether specimen collection was from the first morning void or later, and whether in the previous 24 hr there had been menstrual bleeding or sexual intercourse.

Specimen bottles were 1-oz wide-mouth polypropylene jars with a screw-on lid. Each jar was labeled with the woman's identification number and date of collection. Urine jars were packed eight to a box. Two specimens were collected on Monday mornings, so that each box represents 1 week of urine collection. Our hormone immunochemical assays require relatively small quantities of urine, ranging from 4 mL for hCG to 0.4 mL for steroids. Urine left over after analysis was transferred to borosilicate glass vials for long-term storage.

Human chorionic gonadotropin concentration rises very rapidly during pregnancy, so that cross-contamination from the urines of pregnant women to other urines is a threat. Every effort was taken to avoid such contamination; for example, when women reported that they were pregnant, their boxes of urines were individually stored in zip-lock plastic bags.

We collected daily specimens from a total of 301 women in three categories. Two hundred thirty women were enrolled at the time they discontinued birth control in order to become pregnant (4). Forty women collected urine while using intrauterine devices (IUDs) as their method of contraception (5). Thirty-one women had had tubal ligations for family planning reasons.

These groups of women differed somewhat in their age, pregnancy experience, and education. However, all groups were highly cooperative in collecting urine. Only four women were not able to contribute adequate urine or daily record card information and were therefore excluded. The remaining 297 women contributed urines for 98% of their study-days in each of the three categories. The percent of late voids (urines collected later the same day) was 5% for women using no birth control, 6% for women with tubal ligations, and 7% for women using IUDs.

In order to assess the importance of payments to volunteers, we sent anonymous questionnaires to 80 current and past participants. Seventy women returned the questionnaires. Fifty-three said they probably would not have volunteered for the studies if there had not been payment, and another 10 said they might have lost interest before the end of the study if there had been no payment. Only seven women (10% of the respondents) stated that payment did not encourage their participation. Volunteers were mostly middle-class, college-educated women for whom $10 a week was at most a marginal contribution to family income. We believe that payment helped establish the seriousness of the study, and provided token compensation for the inconvenience to which women were put on the study's behalf. This is similar to conclusions from a study of payment for mail questionnaires (6).

**Urinary Measures of Female Reproductive Hormones**

Serum hormone levels are the most accurate reflection of production for most hormones. Urinary levels of a hormone (or its metabolic products) may be good indicators of endocrine production depending on patterns of metabolism and excretion of the hormone and the stability of the urinary products.

Among reproductive-age women, the production of reproductive hormones is cyclic. It is necessary to make frequent measurements in order to describe the cyclic production of these hormones accurately. One major advantage of urine assays over serum is in the ease of frequent urine collection. Repeated blood sampling is rarely feasible in field studies. Urine specimens offer a further advantage in the case of pituitary hormones that have pulsatile secretion: urine integrates serum levels over a period of time in a way that a single blood specimen cannot.

Regularly scheduled daily urine collection reduces the effects of diurnal variation. For daily urine collections, a schedule of first-morning collection is perhaps the most convenient. First-morning collections also are relatively more standard in their concentration than collections at other times of the day, since there is presumably little or no fluid intake by the volunteer during the night. However, any once-a-day schedule of collection could miss significant portions of hormones that peak at other times of the day, if those hormones are rapidly cleared.

Over the past 4 years we have performed more than 30,000 hormone assays in duplicate or triplicate using nine radioimmuno- and immunoradiometric assays. Several of these assays have been carried out in the laboratories of the investigators who developed them. Assays for hCG have been performed at Columbia University, hLH assays at Columbia and at Louisiana State University, and steroid assays at Atlanta University. Shipments to the laboratories were by overnight freight, with specimens packed in dry ice.

The specific hormones that have been measured are the estrogen metabolites estrone-3-glucuronide (E\textsubscript{1}G) and estradiol-16-a-glucuronide (E\textsubscript{2}G), the progesterone metabolite pregnanediol-3-glucuronide (PdG), hLH (three different assay methods), and hCG (three different assay methods).

We will describe the laboratory methods for these
assays, and then we will discuss the utility of the data from each of these assays for epidemiologic studies.

Laboratory Methods

Assays of E2G and E316G were carried out by direct radioimmunoassay (RIA) procedures using antisera developed in the Atlanta laboratory (7,8). The assay for pregnanediol used an antisemium supplied through the WHO Reagent Matching Program and adopted the general methods described by Samarajewa et al. (9).

Three assays for hLH were used, detecting various combinations of intact hLH and hLH metabolic products. The first assay employed an RIA with Con A Sepharose extraction, as described by Wehmann et al. (10). The two other assays used monoclonal antibody-based two-site immunoradiometric assays (IRMA). Methods for these assays are unpublished and so are described in detail here. Assays were performed in Immulon II microtiter plates with removable wells (Dynatech Inc.). In order to extract intact hLH from urine, monoclonal antibody A105 to the α subunit (11) was adsorbed to the solid phase surface. In the other assay, monoclonal antibody B201, produced against fragments of the β subunit of hCG and having strong cross-reactivity with free hLH β subunit and hLH β subunit fragments, was coated onto microtiter plate wells in conjunction with A105. This latter assay format is described as a total hLH IRMA and detects free hLH β and β fragments in addition to intact hLH. Solid phase-bound hLH, hLH β, and β fragments were detected utilizing monoclonal antibody B105 labeled with 125I (11).

Microtiter plates for two-site IRMAs were prepared as follows. Two hundred microliters of binding buffer (0.2 M NaHCO3, pH 9.5) containing 5 μg A105 and 8 μg B201 (total hLH assay), or 5 μg A105 only (intact hLH assay), were added to each microtiter plate well and incubated for 16 hr at 4°C. Following removal of the antibody solution, the wells were washed once with deionized water. Subsequently, 200 μL of 1% BSA and 0.1% NaAzide in water were added and incubated at room temperature for 4 hr to block excess antibody binding sites. The blocking solution was removed, and the wells washed twice with deionized water. Microtiter plates prepared in this manner could be stored without significant loss of binding activity for up to 4 weeks at 4°C.

Prior to assay, 1-mL aliquots of urine specimens were adjusted to approximately pH 7.4 by the addition of 0.1 mL of 1 M Tris-HCl, pH 9.0. The specimens were then centrifuged at 2000 × g for 15 min at 4°C. Assays were started by the addition of 200 μL aliquots of treated urine specimens and standards in duplicate to the monoclonal antibody-coated wells. Standard preparations of highly purified, intact hLH and free hLH β subunit were obtained from the National Pituitary Agency and dissolved in Buffer A (0.01 M NaHPO4, 0.15 M NaCl, 0.01 M EDTA, 0.1% NaAzide, and 0.1% bovine γ-globulin, pH 7.4). Microtiter plates were incubated at room temperature for 24 hr. Specimens and standards were then removed by aspiration, and the wells washed twice with deionized water. Two hundred microliters of Buffer A containing 40,000 125I-B105 were added to each well and incubated at room temperature for 24 hr. Tracer antibody was then removed by aspiration, and the wells washed five times with deionized water. Individual wells were placed in 12 × 75-mm polystyrene tubes, and the amount of radioactivity determined using a Packard Multiprias gamma counter. Data reduction was performed automatically using a spline fit program.

Human chorionic gonadotropin was measured by three methods. Two of the hCG assays used reagents specific to the carboxyterminal peptide of the β chain: an RIA sensitive to about 0.40 ng/mL (12) and an IRMA sensitive to about 0.02 ng/mL (13). The third and least sensitive assay is an RIA using the SB6 antibody, as modified by Edmonds (14). A detailed comparison of these three hCG assays has been previously published (4).

Applicability for Epidemiologic Studies

Figure 1 shows an example of several urinary hormone profiles obtained from the urines of a healthy woman who was trying to become pregnant. This woman discontinued her birth control just after a menses, at which time she was enrolled and began to collect urine. Menses during the study is indicated by the shaded band.

Starting early in the first cycle, the top panel shows a gradual rise of one of the estrogen metabolites (E2G), reaching a peak in midcycle and then dropping. The second panel shows a peak of luteinizing hormone during the same cycle, following the rise of estrogen. Rupture of the ovarian follicle typically occurs soon after the midcycle surge of hLH.

The third panel shows production of progesterone during the luteal phase, when the ruptured ovarian follicle is converted to a corpus luteum. The fourth panel shows hCG results; no hCG was found in the first cycle. The bottom panel shows days of intercourse. No intercourse occurred during this cycle from the third day before the hLH peak through the third day after.

In the second cycle, the hormone events are repeated. In contrast to the earlier cycle, there was intercourse on the 2 days before and after the hLH peak. This cycle resulted in successful conception and pregnancy, as signalled by the abrupt rise in hCG (fourth panel). This pregnancy ended with delivery of a healthy girl.

As a selected example, this figure illustrates the descriptive power of hormone profiles in combination with menstrual and intercourse data. However, the potential value of these assays for epidemiologic studies is not found in selected examples, but rather in their applicability to large groups of women. For example, an assay that provides interpretable data for only a fraction of women would probably not be suitable for epidemiologic studies, even if the data were very clear for par-
Estrogen and the Urinary Metabolites

Natural estrogens comprise a diverse family of similarly acting hormones. These hormones are produced and metabolized by many different pathways that would not be well summarized in a single measure of estrogen. In nonpregnant women of reproductive age, the ovary is the major source of estrogens. Estrogens also are produced by the adrenals and by peripheral conversion of androgens, particularly in fat tissue. Circulating estrogens include estrone, estriol, and estradiol, which is the most potent.

Estrogens are metabolized in several different tissues (most importantly the liver, but also the intestine, kidney, brain, and blood) to catechol estrogens, glucuronides, and sulfates. Metabolized estrogen is excreted in feces, saliva, and sweat, as well as in urine. Some of the conjugated metabolites enter the gut via the bile, are converted back to free or active estrogens by gut bacteria, and then are reabsorbed into the circulation. This enterohepatic-biliary circulation can have large effects on the levels of serum estrogens, so that neither serum nor urinary estrogen levels are direct quantitative measures of ovarian production. The use of antibiotics, for example, can profoundly affect serum and urine levels of the estrogen metabolites by altering intestinal bacteria.

Several estrogen metabolites are measurable in urine, including glucuronides of estrone, estradiol and estriol, and the catechol estrogens. In most women, over half of the urinary estrogen is in the form of catechol estrogen metabolites. These metabolites are relatively unstable, and urine must be acidified after collection in order to preserve the metabolites for assay.

The next most abundant urinary estrogen metabolite is E$_1$G, which correlates well with serum levels of estradiol (the chief ovarian estrogen). The glucuronide estrogen metabolites are more stable and are reliably measured in urines collected with no special preparation. E$_1$G is the most studied urinary estrogen and pro-
vides a useful measure of ovarian function (15,16). The one additional metabolite that we have assayed, EDG, behaves very similarly to estrone glucuronide.

In general, estrogen patterns are similar within women but can vary considerably between women. This variability may reflect differences in production, metabolism, diet, medication, or other characteristics of the women.

**Progesterone and Its Urinary Metabolite**

Progesterone is the major hormone product of the corpus luteum. The small amount of progesterone made by the adrenals is negligible, so that serum levels of progesterone can be used as a measure of corpus luteum function. Low progesterone levels in the luteal phase of the cycle are clinically used to identify anovulatory cycles.

The major metabolic product of progesterone is pregnanediol glucuronide, which is excreted in urine. Serum progesterone and urinary pregnanediol glucuronide are highly correlated, which makes the urinary measure very useful (17). The first cycle in Figure 1 shows the luteal phase increase of progesterone after ovulation, and a decline just before onset of menses.

The ratio of urinary E2G to pregnanediol glucuronide has been used to identify a midcycle day close to the day of LH peak (18). A rapid decline of the ratio marks the time immediately after the estrogen peak, when estrogen is falling and progesterone is rising. This steroid surrogate for the ovulatory phase of the menstrual cycle is dependably found in all but a few ovulatory cycles.

**Luteinizing Hormone (hLH)**

Luteinizing hormone and follicle-stimulating hormone (FSH) are complex glycoprotein hormones produced by the pituitary gland. These hormones surge at midcycle in response to a gonadotropin releasing hormone surge, which in turn is triggered by rising follicular estrogen. In the absence of assays for urinary FSH, we consider only hLH in detail here. The LH surge stimulates the rupture and luteinization of the ovarian follicle. Ovulation usually follows LH surge within 24 to 56 hr (19). The mean duration of the LH surge has been estimated to be about 48 hr (20), but there may be considerable variability. LH is rapidly metabolized and excreted; its serum half-life is less than 2 hr. We used three measures of urinary hLH and its metabolites in an effort to detect the LH surge reliably, but without great success.

We measured daily urinary LH in 89 cycles with an RIA that detects intact LH and metabolites, 109 cycles with an IRMA for intact LH, and 72 cycles with an IRMA that detects free LH β-subunit and its fragments as well as intact LH. Results were similar in every case: A reasonably clear LH surge could be identified in 68 to 74% of the cycles. In the remainder, there was more than one surge, an ill-defined surge, or no surge at all. The proportion of cycles lacking a definable LH surge was similar whether looking within cycles of individual women or across all women.

We believe that our LH assays are missing real midcycle surges. Most of the cycles with no clear LH surge have patterns of estrogen rise and fall with progesterone rise that suggest the occurrence of normal ovulation. Six babies were conceived in cycles that had no apparent rise in urinary LH with one or more assays, further evidence that real LH events were missed.

There are several reasons why we could be missing LH surges in our urines. Perhaps most likely, an LH surge that lasts for less than 24 hr could be undetected when samples are collected only once a day, or at the wrong time of day. Edwards recently reported data that show a tendency for the LH surge to begin between 7 a.m. and noon (21). Corson found that the LH surge was detectable in 56% of cycles when urine was collected between 5 A.M. and 8 A.M., and in 94% when urines were collected between 11 A.M. and 3 P.M. (22). These data suggest that first-morning urine may be poorly timed for optimum detection of the LH surge of ovulation.

A second possibility is that LH is excreted into urine in many fragments, and our assays may not have broad

![Figure 2](https://example.com/fig2.png)

**Figure 2.** Urinary hCG profile for a healthy woman attempting pregnancy, indicating subclinical pregnancy loss followed by clinically recognized pregnancy.
enough specificity to detect certain varieties of hLH products. Thirdly, even though we are missing only 2% of urine specimens, it is probable that a few of those missing specimens fell on the day of the hLH surge.

The hLH assay methods we have evaluated using first-morning urines have not been particularly practical from an epidemiologic point of view. Each of the assays fails to provide an indication of the ovulatory phase for at least a quarter of all cycles. Assays for hLH in first-morning urines have not been highly successful elsewhere either, although this is not necessarily apparent in the literature. Published data on urinary hLH are sometimes based on selected cycles (often not explicitly stated), or averaged over a number of cycles, which obscures the variability among cycles. The issues of urinary hLH measurement, including timing of specimens collection, deserve more study.

Human Chorionic Gonadotropin

Human chorionic gonadotropin (hCG) belongs to the family of glycoprotein hormones that includes hLH and FSH. Unlike the other glycoproteins, however, hCG is customarily produced not by the pituitary but by the trophoblastic cells of the blastocyst. Thus, hCG is a measure of pregnancy and is the most reliable and thoroughly researched of the biochemical pregnancy measures.

Human chorionic gonadotropin and hLH are structurally similar and act at the same receptor site. This similarity has made it difficult to measure very small quantities of hCG in the presence of hLH. Although several hCG assays have been regarded as β-chain specific, it is only since the development of antibodies specific to the unique carboxyterminal peptide of the hCG β chain that highly specific hCG assays have been possible (13).

Concentrations of hCG in urine and blood are approximately the same (23). hCG is excreted in urine largely in its intact form, and is extremely stable. In a conception cycle, urinary hCG is first detectable about 7 to 10 days after ovulation, near the time of implantation, and several days before the expected onset of menses.

The chemical detection of pregnancy before the pregnancy is otherwise apparent makes it possible to identify clinically unrecognized pregnancy losses. Figure 2 shows an unrecognized pregnancy loss in the second cycle, followed in the third cycle by successful conception and eventual delivery of a healthy girl.

It is essential in studies of early pregnancy loss to use an hCG assay with adequate sensitivity and specificity. Problems with sensitivity and specificity of hCG assays have contributed to inconsistent findings in the literature concerning the frequency of clinically unrecognized pregnancy losses (14,24,25). Those studies did not collect daily specimens and used assays that are susceptible to cross-reaction with hLH. Furthermore, the assays used are too insensitive to detect most of the early pregnancy losses identified by the IRMA (4).

Summary

We have described our experience with the measurement of urinary reproductive hormones in epidemiological studies. We find that it is practical to collect daily urine specimens from healthy volunteers. We also find that the hormone events of the menstrual cycle and early pregnancy are reliably measured by assays of first-morning urine specimens, with the notable exception of the midcycle hLH surge. The estrogen/progesterone ratio may be an adequate surrogate in such samples for detection of the ovulatory phase.

Technical advances in the assay of reproductive hormones will undoubtedly continue. Prospective studies of female reproductive physiology are now possible on a large scale, and will become increasingly practical. Such studies should be able to provide valuable insights in many areas of epidemiologic research, including the biology of fertility and early pregnancy, the sensitivity of reproductive function to disruption by occupational and other environmental hazards, and the relation of reproductive function to eventual risk of chronic disease.

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